THE UNIVERSITY OF ALBERTA

MOLECULAR INTERACTIONS OF $(Na^+ + K^+)$-ATPase WITH OUABAIN AND THE MEMBRANE

by

© ANTHONY FELIX ALMEIDA

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For my daughters Elise, Camille and Diana who were all too young to be aware of their contribution.

ABSTRACT

The membrane bound enzyme ($Na^+ + K^+$)-ATPase has a characteristic sensitivity to temperature such that it is more efficient above a critical temperature (Tc) than below it. This phenomenon is frequently displayed as an Arrhenius plot (log rate v/s 1/T) from which two activation energies Ea_1 and Ea_2 can be derived. This temperature dependence is believed to involve fluidity changes in the membrane lipids.

Lipid modification of ($Na^+ + K^+$)-ATPase enriched membranes from rabbit kidney and from ox brain, was achieved by treatment with deoxycholic acid (DOC), with sodium dodecyl sulfate (SDS), or by lipolysis with phospholipase-A. Changes in the membrane consequent to treatment were examined in three ways:

a) The effects of temperature on the specific activity of the enzyme.

b) The effects of temperature on the binding of [^{3}H]-ouabain to the enzyme.

c) The changes in membrane fluidity using electron spin resonance (ESR).

The following observations were made:

1. Specific activity of ($Na^+ + K^+$)-ATPase

Detergent treatment increased the specific activity of the enzyme, but the non-linearity of the Arrhenius plot remained. Ea_1 and Ea_2 varied slightly, but maintained a constant relationship (Ea_1:Ea_2) of about 0.5.

PPL-A caused an initial increase in specific activity of untreated membranes followed by a progressive decline. Detergent

treated membranes showed only a progressive decline in activity.

2. Ouabain binding to $(Na^+ + K^+)$-ATPase

As the enzyme was purified by detergent treatment, the affinity of the drug for the receptor was reduced. Equilibrium binding was also reduced but to a lesser degree.

Temperature affected the rate but not the equilibrium binding of the drug. Binding rates, when presented in the Arrhenius form yielded a non-linear plot for untreated membranes, but not for detergent treated or PPL-A treated membranes.

Drug binding sites and catalytic sites increased together as the enzyme was purified. In contrast, PPL-A caused a greater loss of catalytic sites than of drug binding sites.

3. ESR studies with the lipid spin probe M 12-NSE

$(Na^+ + K^+)$-ATPase enriched membranes exhibit two binding sites for M 12-NSE which permit unrestricted or restricted motion. These two populations, designated U and R sites respectively, have been characterized. R sites are in the core of the bilayer; the location of U sites is uncertain.

Changes in probe mobility with temperature differ for U and R sites. In the Arrhenius form, mobility changes at U sites are linear while at R sites are non-linear. M 12-NSE at R sites experiences three distinct events at 30°C, 20°C and 10°C, which may be phase transitions and separations; after DOC treatment, two events at about 25°C and 10°C were observed, while after SDS treatment only a single discontinuity at about 18°C was obtained. These differences were not apparent in liposomes made from total lipid extracts of treated and untreated membranes.

ESR studies of $(Na^+ + K^+)$-ATPase also provided evidence for changes in the width of the biomembrane on cooling; in contrast, liposomes made from lipid extracts of these membranes were markedly different.

The following conclusions are drawn:

1. Lipids associated with the drug binding site differ in quality and function from those associated with the catalytic activity.
2. Purification results in an increased homogeneity of lipids associated with the enzyme.
3. The enzyme exhibits intimate protein-lipid interactions.
4. It is possible that the energy transduction mechanism of the sodium-pump incorporates the potential energy of an elastic membrane.

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I doff my cap in the direction of the Chemistry Department and remember that I used their ESR spectrometer; and while in this position of respectfulness, I salute Dr. R.E.D. McClung who began at the very beginning and taught me the essentials of electron spin resonance spectroscopy.

Many others have helped in one way or another and I thank them collectively; but Laurie Simonson I thank singularly.

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INTRODUCTION

General

Interest in the cardiac glycosides, a group of drugs of which digitalis is the most widely known, developed along two lines, namely the clinical and the biochemical. Withering is generally credited with having introduced digitalis to the practice of medicine in 1785, but it was not until 1953 that Schatzmann reported the presence of a cation pump in red blood cells that could be inhibited by ouabain. The pump was later shown to be the sodium-plus-potassium-activated-adenosine triphosphatase ($Na^+ + K^+$)-ATPase. According to Withering (1783) digitalis was a drug with very wide application but in 1940, Gold and Cattell showed the inotropic effect to be a consequence of the direct action of digitalis on the myocardium. The three areas, namely the drugs, the clinical pharmacology, and the biochemical pharmacology will be discussed separately in the introduction to this dissertation, but this subdivision does not imply that these areas are unrelated.

Drugs

The cardiac glycosides are composed of an aglycone or genin and a sugar. The sugar moiety may vary in number from one to four molecules, and also in type. Although pharmacological activity is confined to the aglycone, the sugars may influence water solubility and drug penetrability into cells. The suggestion has also been made that the sugar may occupy a subsite on the receptor for these drugs (Erdmann and Schoner, 1974). A disaccharide increases the affinity of the drug, but a trisaccharide decreases it; thus, some structural specificity of the molecule may also exist for the receptor subsite. Qualitatively the different cardiac glycosides are similar in their pharmacological effects.

In vitro experiments generally utilize ouabain as it exhibits greater aqueous solubility.

The pharmacological similarity of these drugs is also manifested in their chemical structure, which is basically a steroid nucleus with the glycoside linkage at the C_3-OH position and an unsaturated five or six membered lactone ring at C_{17}. Saturation of the lactone ring reduces the activity by an order of magnitude while opening of the ring abolishes the activity altogether. A secondary OH at C_3 and an OH at C_{14} are believed to be essential components of stereochemical configuration. The cis A:B isomer is also an essential structural requirement in that the trans A:B isomer suffers a fifty-fold loss of binding (Erdmann and Schoner, 1974).

Clinical Pharmacology

The clinical effects of the cardiac glycosides whether beneficial or toxic are qualitatively similar. The drugs affect the mechanical as well as the electrical properties of the heart. When used in the patient with a congested heart, the beneficial effects are a consequence of the improved force of contraction of the ventricle, that is, the positive inotropic effect. It is not yet clear whether the inotropic response and the toxic effects are mediated through distinct receptors. Indeed the contrary might be true, with the two effects reflecting a difference only in magnitude. The relationships between lethal doses of the drugs on the one hand and I_{50} for the inhibition of $(Na^+ + K^+)$-ATPase on the other was observed by Repke in 1963, and it led him to suggest that the enzyme might be the digitalis receptor.

However the precise role of $(Na^+ + K^+)$-ATPase in the inotropic effect of digitalis on the heart remains a mystery. Essentially

most investigators (Schwartz, 1976; Akera, 1977) have approached the problem from the beginning or the end of the following reaction sequence:

D (drug) + R (receptor) → DR → Inotropic effect.

The studies on drug receptor interactions have sought to establish a correlation between binding of the drug to $(Na^+ + K^+)$-ATPase and its inotropic effect on the heart. These investigations have included whole animal experiments as well as isolated hearts and strips of cardiac tissue. The following is a brief summary of the findings:

(1) Only cardiotonic steroids display enzyme inhibition (Repke, 1961; Matsui and Schwartz, 1967; Matsui and Schwartz, 1968; Albers *et al.*, 1968).

(2) By using experiments that combine *in vivo* and *in vitro* methods, the development of the inotropic response was shown to correlate with drug binding to the $(Na^+ + K^+)$-ATPase (Besch *et al.*, 1970).

(3) Also from combination experiments of the type described above, the observation was made that the drug either did not bind to or was easily dissociated from mitochondria and from sarcoplasmic reticulum (Allen *et al.*, 1975; Jones *et al.*, 1976).

(4) The cardiac glycosides exhibit a range of sensitivities. Beef, human, and dog hearts are at the sensitive end and rats on the insensitive end of the scale (Detweiler, 1967). Rabbits occupy an intermediate position. Inhibition of $(Na^+ + K^+)$-ATPase preparations from different species shows a correlation between the sensitivity of the species and the I_{50} which was time dependent. In the case of the rat the I_{50} was both lower and not time dependent (Akera *et al.*, 1969; Albers *et al.*, 1968; Allen and

Schwartz, 1969; Erdmann and Schoner, 1973a; Schwartz *et al.*, 1969).

(5) The affinity of [^{3}H]-ouabain binding to ($Na^+ + K^+$)-ATPase preparations from different animals showed correspondence with their drug sensitivity. That is, high sensitivity coincided with high affinity of the ($Na^+ + K^+$)-ATPase for the drug (Repke, 1961; Albers *et al.*, 1968; Schoner *et al.*, 1972).

(6) On the species sensitivity scale, dissociation of the ($Na^+ + K^+$)-ATPase-drug complex correlated positively with reversal of the pharmacological effect on isolated hearts as achieved by washing out the drug (Akera *et al.*, 1972).

(7) Some reports contradict the correlations discussed above (Okita, 1973; Okita *et al*, 1973; Roth-Schecter *et al.*, 1970; Dutta and Marks, 1969). They are however considerably weakened by the following: (a) the test animal was the rabbit, which occupies an intermediate position on the species-sensitivity series (Dutta and Marks, 1969; Okita, 1973; Okita *et al.*, 1973), (b) the low ($Na^+ + K^+$)-ATPase activity of the control membrane preparations (Dutta and Marks, 1969; Roth-Schecter *et al.*, 1970; Okita, 1973; Okita *et al.*, 1973) and (c) use of the drug strophanthidin-3-bromoacetate which differs from digitalis in its binding specificity (Dutta and Marks, 1969; Hokin, 1969; Fricke and Klaus, 1971).

Thus, the information from drug-receptor interactions favour some manner of ($Na^+ + K^+$)-ATPase involvement in the inotropic action of these drugs.

At the other end of the sequence (p. 4), the workers have begun with the very reasonable premise that the increased force of contraction resulting from treatment with these drugs, proclaims an altered

calcium pool(s). It is an established fact that the calcium (Ca^{++}) in beating hearts is increased after treatment with cardiac glycosides (Gold and Cattell, 1940; Lee and Klaus, 1971; Marks and Weissler, 1972; Smith, 1973; Langer, 1974), but further characterisation of this phenomenon has not been attained. It is unlikely that digitalis affects Ca^{++} pools associated with the mitochondria or the contractile proteins (Lee and Klaus, 1971).

It is known that Ca^{++} inhibits the $(Na^+ + K^+)$-ATPase in the presence of sodium ions (Na^+) and potassium ions (K^+) (Skou, 1957, Skou, 1965), but in the absence of Na^+, or with low Na^+, the enzyme may be activated (Schon *et al.*, 1972). Finally the suggestion has been made that the affinity of Ca^{++} for some phospholipid in the $(Na^+ + K^+)$-ATPase preparation may be altered by ouabain (Gervais *et al.*, 1975).

The information from studies of Ca^{++} involvement in the inotropic effects suggests a number of possible mechanisms. Three of these postulates have been presented by Schwartz (1976) in a recent review and are listed below:

a) Inhibition of the $(Na^+ + K^+)$-ATPase, leads to increased intracellular Na^+ and consequent increase of intracellular Ca^{++} (Baker, 1972; Langer, 1973; Reuter, 1974).

b) Inhibition of $(Na^+ + K^+)$-ATPase by cardiac glycosides leads to altered Ca^{++}-affinity inside the cell.

c) Inhibition of $(Na^+ + K^+)$-ATPase may lead to loss of K^+ causing a K^+ directed influx of Ca^{++}.

Nevertheless it must be emphasized that postulates are as yet speculative and the final statement must remain that the specific binding of Ca^{++} to the $(Na^+ + K^+)$-ATPase during the inotropic effect has not been demon-

strated (Schwartz, 1976).

Biochemical Pharmacology

1. General

The majority of animal cells possess a high intracellular concentration of K^+ and a low concentration of Na^+. To maintain this state, a so-called "coupled pump" mechanism is necessary, so that both K^+ and Na^+ can be moved simultaneously against their concentration gradients but in opposite directions. After the discovery of a cation pump in red blood cells by Schatzmann (1953), there was a tremendous drive to locate an enzyme that would fill the role of the pump. In 1957 Skou described such an enzyme in fragmented nerve membranes from the walking legs of crabs. This enzyme required Na^+ and K^+ for full activation and utilized adenosine triphosphate (ATP) as its substrate. It was found to be a sodium-plus-potassium-dependent magnesium ATPase now known as $(Na^+ + K^+)$-ATPase. Thus two associated phenomena can be defined, namely the derivation of chemical energy from ATP by the enzyme and its utilization by the pump for physical work. The similarities of the two processes are now well recognized, and have been extensively reviewed (Wheeler and Whittam, 1970; Skou, 1971) particularly in an article by Glynn (1968). Some of the similarities between the pump and the enzyme are as follows:

i. The pumping of Na^+ from within the red cell occurs only if K^+ is present on the outside of the cell. This simultaneous presence of Na^+ and K^+ is also a requirement for the $(Na^+ + K^+)$-ATPase activity of broken membrane preparations.

ii. Inhibition of Na^+ and K^+ pumping on the one hand and $(Na^+ + K^+)$-

ATPase activity on the other, occur at similar concentrations of the cardiotonic glycosides.

iii. The structure-activity relationships of the cardiac glycoside molecules are common to both transport inhibition as well as $(Na^+ + K^+)$-ATPase inhibition.

iv. K^+ and ouabain compete in both mechanisms.

v. The optimal concentration of K^+ and of Na^+ is the same for both processes.

vi. Both processes display vectorial qualities, with a requirement for Na^+ and ATP inside the cell and K^+ and ouabain outside the cell.

This evidence clearly shows that the transport mechanism and the $(Na^+ + K^+)$-ATPase activity are closely related phenomena. Thus it is currently accepted that the transport of Na^+ and K^+ in many cells is a process that derives energy directly from the hydrolysis of ATP via $(Na^+ + K^+)$-ATPase.

2. Catalytic aspects

For full activation this enzyme requires magnesium ions (Mg^{++}), Na^+, and K^+, and is inhibited by the cardiac glycosides. In the absence of Na^+ and K^+, membrane hydrolysis of ATP still occurs, but at a greatly reduced rate. This component, which requires Mg^{++} but not Na^+ or K^+ for activation is variously referred to as Mg^{++}-ATPase, ouabain insensitive ATPase or basal ATPase. In this dissertation, the term $(Na^+ + K^+)$-ATPase will be used to indicate that component of membrane ATP hydrolysis which depends on Mg^{++}, Na^+, and K^+, and which is completely inhibited by ouabain.

The hydrolysis of ATP by the $(Na^+ + K^+)$-ATPase is a complex process and thus presents a number of facets for investigation. Thus,

the substrate specificity, the characteristics of the cations and the inhibition by ouabain have all been examined extensively. This has generated a vast amount of information, but a complete understanding has not yet been achieved. The following reaction mechanism, although incomplete, is reproduced from Schwartz (1976) to facilitate discussion of the different facets of the problem.

$$E + Mg^{++}\text{-ATP} \xrightleftharpoons{Na^+_i} (E_1 - P)\text{-}Na + ADP$$

$$(E_1 - P)\text{-}Na \xrightleftharpoons{(Mg^{2+})?} (E_2 - P) + Na_o$$

$$H_2O + E_2 - P \xrightleftharpoons{K^+_o} E_2 - K + Pi$$

$$E_2K \rightleftharpoons E_2 + Ki$$

$$E_2 \xrightleftharpoons{ATP} E_1$$

ATP is the most effective of the nucleoside triphosphates (Charnock *et al.*, 1963), with CTP (Hokin and Yoda, 1964; Matsui and Schwartz, 1966; Towle and Copenhaver, 1970) and ITP (Matsui and Schwartz, 1966; Towle and Copenhaver, 1970) being less effective and GTP (Matsui and Schwartz, 1966; Schoner *et al.*, 1968) only marginally so. The enzyme does not display ouabain-sensitive hydrolysis of UTP (Hokin and Yoda, 1964). The 6-amino group of the purine ring and the γ-phosphate are deemed essential for tight binding and Mg^{++}-ATP appears to be the true substrate for this enzyme (Hexum *et al.*, 1970), although work by Hegyvary and Post (1971) casts some doubt on the latter statement. There is ample evidence to show that the enzyme-nucleoside bond is an acylphosphate (Nagano *et al.*, 1965; Hokin *et al.*, 1965; Alexander and Rodnight, 1970), which has now been characterized as β-aspartylphosphate

(Post and Orcutt, 1973; Post and Kume, 1973; Hokin, 1974; Degani *et al.*, 1974).

The cation requirements of this enzyme have been studied extensively. Hexum *et al.* (1970) have shown from studies of catalytic rates that Mg^{++}-ATP is the substrate for this enzyme, and free ATP or free Mg^{++} results in competitive and noncompetitive antagonism. Thus, not only is Mg^{++} essential for the enzyme, it must also be maintained at a concentration about equal to the ATP. Other divalent cations, namely manganese and cobalt can replace Mg^{++} but the over all efficiency of the hydrolytic reaction is reduced by 90% (Sachs and Welt, 1967; Atkinson *et al.*, 1968; Atkinson and Lowe, 1972).

The interaction between the sodium pump and the two transported cations Na^+ and K^+ can be discussed under five different categories or sub-headings as was recently described by Glynn and Karlish (1975).

1. Na^+-K^+ exchange
2. Reversed Na^+-K^+ exchange
3. Na^+-Na^+ exchange
4. K^+-K^+ exchange, and
5. Na^+efflux without exchange.

The first of these five modes, the Na^+-K^+ exchange is the normal or forward mode of operation of the sodium pump, while the remainder are achieved only by manipulation of the operating conditions. Although the investigations of these so called partial reactions of the pump (Glynn *et al.*, 1971) have contributed valuable information, they are not considered pertinent to this dissertation and will not be discussed.

Under normal physiological conditions the sodium pump in a variety of tissues including human red blood cells, nerve and muscle, display a similar stoichiometry (Bonting and Caravaggio, 1963; Sen and Post, 1964; Baker, 1965; Whittam and Agar, 1965; Garrahan and Glynn, 1967; Dudding and Winter, 1971; Garay and Garrahan, 1973; Ellory and Carleton, 1974). For every molecule of ATP that is hydrolyzed, three Na^+ are pumped out in exchange for two K^+. The sodium pump would therefore appear to be electrogenic when operating in its normal mode. However, the suggestion has been made that a proton from water may move inward with the potassium ion thereby making the pump electrically neutral (Schwartz *et al.*, 1975).

The studies undertaken to investigate interactions between cations and the $(Na^+ + K^+)$-ATPase are mostly kinetic in nature, and employ enzyme enriched membrane fragments. Although often vesicular in form, these membrane fragments do not maintain ionic gradients. Therefore, extrapolation of the information to a sodium pump in an intact cell may not be valid. It is generally accepted that there are three sodium sites on the inside and two potassium sites on the outside of the cell membrane. Using a brain membrane preparation, Schwartz' group have shown that the apparent affinity of the sodium sites but not the potassium sites is equivalent (Lindenmayer *et al.*, 1974). Different affinities for potassium were also reported for erythrocytes by Garahan and Glynn (1967) and by Ellory and Carleton (1974).

The Na^+:K^+ ratio is optimal over a range between 5:1 and 10:1. For normal function the pump has an absolute requirement for sodium (Dahl and Hokin, 1974), but a variety of cations will substitute for potassium (Britten and Blank, 1968; Skulskii and Manninen, 1973), to

achieve different degrees of efficiency.

3. Ouabain binding studies

The reaction sequence shown previously (page 8) depicts two phosphorylated intermediates. Considerable evidence exists to support this concept and the transformation of the E_1-P state to a lower energy E_2-P state is currently well accepted (Charnock *et al.*, 1963; Rose, 1963; Charnock and Post, 1963; Albers *et al.*, 1963; Hokin *et al.*, 1965; Kanazawa *et al.*, 1967). Mg^{++} as well as Na^+ are required for this conversion and in the presence of potassium ions the E_2-P complex is dephosphorylated (Post *et al.*, 1965). It is also generally accepted that the two states represent two conformations of the phosphorylated enzyme (Post *et al.*, 1969; Post *et al.*, 1972). Despite its general acceptance, the role of this conformation change in the translocation of Na^+ and K^+ has not been explained in any detailed molecular way.

In 1968 it was reported that the $(Na^+ + K^+)$-ATPase could be phosphorylated in the presence of inorganic phosphate (Pi) and Mg^{++} (Albers *et al.*, 1968; Lindenmeyer *et al.*, 1968). Sodium inhibited the formation of this particular E-Pi complex but K^+ had no effect (Lindenmeyer *et al.*, 1968). suggesting that E-Pi is not identical to the E_2-P complex referred to above. Work from a number of different laboratories, applying a variety of methods, supports the concept that the E_2-P differs from the E-Pi (Lindenmeyer *et al.*, 1968; Hart and Titus, 1973a; Hart and Titus, 1973b; Akera *et al.* 1974; Schwartz *et al.*, 1974).

The binding of ouabain to both complexes has been extensively studied, although inhibition of the enzyme activity necessitates binding to E_2-P. Two underlying questions have prompted workers to seek a

better understanding of the phospho-enzyme complexes:

i) Will information on the ouabain-enzyme interaction reveal molecular characteristics of the sodium pump?

ii) Will the pharmacological actions of these drugs, namely the therapeutic and the toxic effects, be better understood by examining the binding of ouabain to the enzyme?

The interaction of cardiac glycosides with the $(Na^+ + K^+)$-ATPase has been approached from two points of view, namely the potency of enzyme inhibition and the stoichiometry of binding. As mentioned previously the inhibition of enzyme activity shows good correlation with the inotropic effect that is achieved with these drugs. That is to say, noncardiotonic glycosides do not inhibit the enzyme, and furthermore only the cardiotonic glycosides compete for this membrane receptor (Matsui and Schwartz, 1967; Matsui and Schwartz, 1968; Schwartz *et al.*, 1968; Albers *et al.*, 1968). In addition it has been reported from kinetic studies that dissociation of the ouabain-enzyme complex may be related to species sensitivity (Allen and Schwartz, 1969; Tobin and Brody, 1972). At present these observations cannot be used to further our understanding of the mechanism of action of the beneficial or of the toxic effects of these drugs.

Quantitative measurements of binding data are markedly handicapped by the absence of a specific reference component. Consequently, stoichiometric values have been derived with respect to the phosphorylated intermediate (Albers *et al.*, 1968; Jorgensen, 1974a), the enzyme hydrolytic activity (Albers *et al.*, 1968; Jorgensen, 1974a,b), and the submacromolecular structure of the $(Na^+ + K^+)$-ATPase (Kyte, 1972a; Lane *et al.*, 1973; Hokin *et al.*, 1973; Jorgensen, 1974). A further weakness

in such attempts at quantification is the possibility of non-specific binding and the species variation which may occur. Nevertheless some attempts have been made to overcome these difficulties and a brief discussion of the findings is pertinent to this dissertation.

A number of workers have relied on enzyme purification to overcome the aforementioned problems. Despite the use of different methods of purification, results from their laboratories show good correlation. Irrespective of the source of the enzyme, two major polypeptides have been identified as components of the purified enzyme macromolecule (Kyte, 1972a,b; Lane *et al.*, 1973; Hokin *et al.*, 1973; Jorgensen 1974). The larger of these two chains is reported to have a molecular weight of about 95000 as determined by SDS-polyacrylamide-gel electrophoresis (Kyte, 1971a,b; Kyte, 1972b; Hokin *et al.*, 1973; Lane *et al.*, 1973; Jorgensen, 1974; Uesugi *et al.*, 1971), and 139000 by gel filtration (Kyte, 1972b). This polypeptide carries the site for phosphorylation during ATP hydrolysis (Alexander and Rodnight, 1970; Uesugi *et al.*, 1971; Kyte, 1971a,b; Avruch and Fairbanks, 1972; Collins and Albers, 1972; Hart and Titus, 1973b). Ouabain must also bind to this subunit, since it is known to bind to the E_2-P complex. By examining the reactivity of the sulfhydryl groups Hart and Titus suggested that the binding sites for Na^+ and K^+ may also reside on the large polypeptide (Hart and Titus, 1973).

The smaller subunit is a sialoglycoprotein with a molecular weight of about 55000 (Kyte, 1971; Uesugi *et al.*, 1971; Kyte, 1972a,b; Lane *et al.*, 1973; Hokin *et al.*, 1973; Hokin, 1974). This smaller polypeptide has not been linked to any catalytic function.

Nevertheless it does undergo purification in parallel with the larger polypeptide, the catalytic unit. It is believed that the two polypeptides exist in some mass ratio which has not yet been established. Some of the values quoted for this ratio are 1.7 (Kyte, 1972b), 1.9, (Lane *et al*, 1973), 2.3 (Hokin *et al*, 1973), and 2.8 (Jorgensen, 1974), and the discrepancies may be due to the variation in the preparative procedures and/or the determination of the size of the polypeptide and/or the species from which the enzyme was derived.

The information obtained from ouabain binding studies cannot be collated easily, as it is often varied and contradictory. In 1968, Lindenmayer *et al*, demonstrated the presence of the E-Pi-Ouabain (E-Pi-O) complex, and suggested that its conformation was different from that of the E_2-P-O complex. Currently this view is also held by other workers (Hart and Titus, 1973a; Hart and Titus, 1973b; Akera *et al.*, 1974; Schwartz *et al.*, 1974). The suggestion implies that the receptor species is the same, and a number of observations would support this view. For example, the level of drug bound in the presence of different ligands did not result in different values (Inagaki *et al* 1974). Furthermore, dissociation of the complexes with unlabelled drug was also the same (Schon *et al.*, 1970, Lane *et al* 1973) and finally, the activation energies derived from rate constants for the binding of ouabain to these two enzyme-phosphate complexes were not different at 21 and 23 kcals/mole respectively (Wallick and Schwartz, 1974).

In contrast to the work of Inagaki *et al.* (1974), several workers have reported that the maximal drug bound to the two complexes is not the same but higher in the E_2-P-O form (Schwartz *et al.*,1968; Albers *et al.*, 1968; Skou *et al.*, 1971).

Studies have also been undertaken to examine the relationship between drug binding and specific activity. As the specific activity of the enzyme is increased, the number of binding sites also increase. This correlation between maximal binding and specific activity, although supported by work from a number of different laboratories (Schwartz *et al.*, 1968; Albers *et al.*, 1968; Hansen, 1971; Erdmann and Schoner, 1973b), is not unchallenged. Reports also claim that the capacity of the enzyme for ouabain binding and E_2-P formation cannot be correlated (Matsui and Schwartz, 1967; Matsui and Schwartz, 1968; Schwartz *et al.*, 1968; Albers *et al.*, 1968).

Attempts have also been made to study the effects of ouabain binding to preparations subjected to lipase treatment. Some reports show a linear correlation between loss of specific activity and ouabain binding, but others reject this correlation. Goldman and Albers (1973) found that after treatment of the enzyme from eel electroplax membranes with phospholipase-A (PPL-A) the specific activity and the ouabain binding capacity decreased in parallel if the E_2-P-O was the complex estimated. Erdmann and Schoner (1973b) using ox brain ($Na^+ + K^+$)-ATPase found that under conditions favourable to E-Pi-O formation PPL-A caused a loss of the catalytic activity, both the ($Na^+ + K^+$)-ATPase and the phosphatase (K^+-pNPPase, E.C.3.1.1.4), but the ouabain binding and ^{32}P labelling of the membranes remained. If the membranes were treated with phospholipase-C (PPL-C) however, the ouabain binding was not impaired in parallel with the specific activity (Goldman and Albers, 1973).

From the aforementioned information, it would seem likely that the protein component or receptor macromolecule is a single species

as was suggested by Schwartz' group (1968), however the site of ouabain binding probably differs in the E_2-P-O and the E-Pi-O complexes. These two sites may be maintained by different conformations of the protein. It is not clear at present just how extensive these differences might be. However it is certain that the protein macromolecule is the same for both, and the differences may reside at the tertiary and quarternary levels of organisation. Some information has been gained from the effects of potassium on the binding of ouabain to membrane preparations containing ($Na^+ + K^+$)-ATPase. In particular, these studies have attempted to assess the occurrence of conformation changes in the reaction sequence of the enzyme. Robinson (1970) proposed a K^+-directed conformational change at the step involving the cleavage of the enzyme-phosphate bond. In a subsequent publication Charnock *et al.* (1971) also postulated a K^+-induced conformational change to facilitate release of Pi from the enzyme macromolecule. Akera and his co-workers (1974) showed quite definitely that K^+ has a distinct influence on the dissociation of the two complexes. K^+ stabilizes the E_2-P-O complex but not the E-Pi-O complex. In apparent contrast, K^+ also reduces the rate of ouabain binding to E_2-P. They proposed that K^+ induces a conformational transition which alters the ouabain site thereby decreasing its affinity for ouabain. This K^+-induced conformational change is therefore independent of dephosphorylation. They also suggest that there may be two different conformational states or a single state with two different ouabain binding sites, which lead to formation of the E_2-P-O or the E-Pi-O complex.

The following is a summary of the preceding discussion:

1) The E_2-P-O and the E-Pi-O complexes are similar in some respects and very different in others.

2) Increasing specific activity of membrane preparations may or may not be correlated with increased formation of E_2-P-O.

3) The loss of specific activity by treatment with PPL-A may or may not correlate with decreased formation of E_2-P-O, but does not correlate with E-Pi-O. When the membranes are treated with PPL-C, the loss of specific activity is not accompanied by a parallel loss of E_2-P-O formation.

This summary emphasizes the complexity of the $(Na^+ + K^+)$-ATPase macromolecule. While it is clear that it is capable of undergoing conformational changes (at least two of which have been established), little direct biophysical evidence exists to support this view. Each conformer is endowed with an assortment of binding sites for the different ligands associated with the enzyme function. Furthermore these binding sites may influence each other. This is especially true of the sites for Pi, K^+ and ouabain. Finally, the phospholipids of the bilayer in which the macromolecule is embedded may participate in one or more of the conformations. This involvement of the lipid is the topic of the following discussion.

4. Lipid involvement

Arrhenius plots (Arrhenius, 1889) of data obtained from enzyme function are often used to demonstrate the thermal sensitivity of the $(Na^+ + K^+)$-ATPase. Such a plot of the reciprocal of the absolute temperature versus the logarithm of the enzyme activity usually results in a discontinuous line with a break at a so-called "critical temperature" (Tc) near 20°C. Above the critical temperature the slope of the line is less than below it. Thus the enzyme has a lower energy of activation above Tc and higher energy of activation below it.

In order to understand the discontinuities referred to above,

researchers have generally directed their investigations towards a better understanding of the qualitative and quantitative characteristics of the phospholipids associated with this enzyme. A vast amount of information has accumulated and it is discussed under the four following sub-headings:

a) Reactivation of delipidated membranes with respect to specific activity as well as phospholipid specificity.

b) Analysis of the phospholipids of $(Na^+ + K^+)$-ATPase enriched membranes.

c) The effects of temperature and/or lipid depletion on the partial reactions.

d) Reconstitution of lipid-free solubilized $(Na^+ + K^+)$-ATPase enzyme preparations into liposomes.

a) Lipid depletion is generally achieved by treatment with lipases, detergents or organic solvents. Of the lipases A,C, and D, phospholipase-A (PPL-A) has been used most often. Amongst the detergents, the nonionic detergent Lubrol-WX and the anionic detergent deoxycholic acid (DOC) are most commonly employed. Organic solvent extraction is relatively rarely used.

Ohnishi and Kawamura (1964) are credited with being the first to demonstrate the implication of a lipid in the mechanism of action of this enzyme, and from their work, they concluded that phosphatidyl serine might be the essential lipid.

Since then a variety of phospholipids have been used to restore the hydrolytic activity to the lipid depleted enzyme. Taniguchi and Iida (1972) achieved reconstitution of a PPL-A treated enzyme with phosphatidyl serine (PS) and with phosphatidyl inositol (PI). Hokin

and Hexum (1972) confirmed this work and extended it to include phosphatidic acid (PA) and didodecyl phosphate as compounds capable of reactivating the enzyme activity. These workers also showed that the loss of activity after treatment with PPL-A was not due to lysophosphatides. Using rabbit kidney cortex, [1] Charnock *et al* (1973) compared the effects of temperature on enzyme enriched membranes after treatment with nonidet-P 40 a nonionic detergent, DOC an anionic detergent, phospholipase-C (PPL-C) from Clostridium Welchii, and bee venom PPL-A. Both detergents and PPL-C showed a small effect on the slope of the line at the lower temperatures, but the discontinuity in the Arrhenius plot remained, and the critical temperature was similar to that observed with untreated membranes. Treatment with PPL-A however, abolished the discontinuity, and this resulted in a single straight line having a unique slope distinct from the earlier values for Ea_1 and Ea_2. Addition of PS to this phospholipase treated enzyme restored the bend, which also occurred at the original Tc. The work also included an examination of the temperature sensitivity of the Mg^{++}-ATPase, the temperature sensitivity of this enzyme was neither biphasic nor linear but curvilinear. Furthermore, this curvilinear plot remained unchanged after treatment with detergents or with lipases. We concluded that the Mg^{++}-ATPase and the $(Na^+ + K^+)$-ATPase are distinct entities even though they may be closely associated in the membrane. We also suggested that the lipids associated with the two enzyme were not the same. The detergent studies showed that although the specific activity could be varied considerably,

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[1] Charnock, J.S., Cook, D.A., Almeida, A.F., and Rebecca To. (1973) Arch. Biochem. Biophys. 159, 393. This work will not appear either in the methods section or in the results section. For completion, a copy is included in the appendix.

the resultant enzyme preparations with an increased number of active centres did not demonstrate any significant change in the thermodynamic parameters as determined by temperature studies. The results from studies with PPL-A confirmed those of others (Ohnishi and Kawamura, 1964; Taniguchi and Iida, 1972; Hokin and Hexum, 1972); the data did not permit any unequivocal statement regarding either the specificity of PS or its location in the membrane matrix with respect to the (Na^+ + K^+)-ATPase.

In addition to PS, PI and PA as lipids capable of restoring the activity and the thermal sensitivity to the lipid depleted enzyme, other compounds have also been used successfully. Kimelberg and Papahadjopoulos (1972) and Palatini *et al* (1972) independently and concurrently reported reconstitution with phosphatidyl glycerol (PG). It is of note that although PC has been used to reactivate delipidated enzyme preparations, it is generally less effective than any of the others that have been used (Kimelberg, 1976). The one exception is the report by Tanaka and Strickland (1965), but this has been criticised on the grounds that the PC was contaminated with PS (Fenster and Copenhaver, 1967). Finally it must be noted that there is a requirement for cholesterol if lipid depletion is effected by the use of organic solvents (Noguchi and Freed, 1971; Jarnefelt, 1972). Thus it would appear that although some lipid is essential for activity a specific phospholipid may not be crucial for enzyme function. In a recent review, Kimelberg (1976) has tabulated the lipids that have been used in reactivation studies. From this table it is apparent that reactivation is most often achieved with PS, and the possibility that this phospholipid participates in some specific manner must still be

entertained. Nevertheless it is certain that removal of lipid from $(Na^+ + K^+)$-ATPase results in a corresponding loss of enzyme activity . (Erdmann and Schoner, 1973b; Stahl, 1973), and this loss can be restored to varying degrees by the addition of phospholipid. Complete removal of lipid leads to an irreversible loss of activity (Hegyvary and Post, 1969; Roelofsen *et al*, 1971; Erdmann and Schoner, 1973b).

The most direct evidence for the involvement of lipids in $(Na^+ + K^+)$-ATPase has been obtained by the use of physical methods employing membrane probes. In 1973 Grisham and Barnett employed a lipid spin probe, to examine the effects of temperature on the membrane lipids. This method which reports fluidity changes in the membrane showed a biphasic response to temperature irrespective of whether the probe was located in microsomes or liposomes made from lipid extracts of $(Na^+ + K^+)$-ATPase. Moreover the values for critical temperature (Tc, cf. p.17) were also identical in these two situations and similar to the Tc determined from an Arrhenius plot of enzyme specific activity. Thus it appears that a phase transition of the membrane lipids is directly involved in the discontinuity observed in Arrhenius plots of specific activity. Since the activation energy for hydrolytic function is increased at temperatures below Tc, it is tempting to suggest that below Tc, the lipids hinder the function of the protein macromolecule. In a similar approach Charnock and Bashford (1975) employed fluorescent probes and were able to confirm the findings of Grisham and Barnett (1973). Furthermore, they avoided the criticism of probe interference in membrane function, by duplication of the liposome study using a light scattering technique. Thus it is currently well accepted that $(Na^+ + K^+)$-ATPase has an absolute requirement for some phospholipid(s).

From the varied results that have been discussed, it is not possible to sustain or reject the concept of a specific phospholipid as essential for reactivation of $(Na^+ + K^+)$-ATPase activity. Roelofsen and Van Deenen (1973) have lisited discrepancies that could accomodate some of the variation, and these have been listed below:

1. The phospholipases used for lipolysis may have been impure, and would consequently lack substrate specificity.
2. The phospholipids used for reactivation may have been impure.
3. Information on residual lipids in the delipidated membranes was absent or sparse; when present the lipids were not characterized.
4. Both the degree of inactivation and reactivation was often neglected.

Although these criticisms certainly apply in a number of instances they do not offer a complete explanation for the discrepancies found in the literature.

Kimelberg and Papahadjopoulos (1974) who also noted discrepancies approached the problem differently. After delipidation of rabbit kidney membranes with DOC they monitored the specific activity and the Tc on the addition of phospholipids which varied both in head group and chain length. Their results showed that PS and dioleoyl-PG, two phospholipids with unsaturated chains produced the highest level of reactivation, PS being the better of the two. In contrast dimyristoyl-PG and distearoyl-PG succeeded only partially in restoring activity. Their temperature study revealed that the control membrane preparation had a Tc of about 30° C which is somewhat higher than that frequently reported by other workers. The delipidated preparation showed no discontinuity in the temperature-activity relationship, and the addition of PS to these membranes introduced a Tc at 15°C. Dioleoyl-PG acti-

vated the delipidated preparation which maintained its linear appearance albeit with a lower activation energy. The effect of chain length was clearly demonstrated by reactivation with dimyristoyl-PG, dipalmitoyl-PG and distearoyl-PG: the longer the methylene chain the higher the Tc. They concluded that a specific requirement for PS and PG was strongly indicated. They also noted that the discontinuities reflected by the enzyme hydrolytic activity did not correspond with the endothermic transitions as determined by differential scanning calorimetry. This is an important observation in the application of physical techniques to biological investigation.

From the data in that paper an interesting observation can be made. Since the delipidated enzyme retained activity, it presumably also retained some lipid. If the retained lipid is the critical lipid then a further addition of other lipids should not have altered Tc. If residual detergent is not a factor in these findings, then another interpretation must be considered; that is, the closely bound lipids are not uniquely and exclusively responsible for the discontinuity in the temperature activity relationship, but the lipids in the membrane matrix may also be determinants of this phenomenon. This introduces the question as to the role of phospholipids in the function of $(Na^+ + K^+)$-ATPase and their contribution to the thermally induced discontinuity.

The phase transitions observed on cooling lipid bilayers has been extensively examined. Although the involvement of the methylene chains of the phospholipids has been known for over a decade (Chapman *et al.*, 1967), a clear understanding of phase transitions in molecular terms is still not possible. In a recent review Lee (1977) reminds us that above the transition the arrangement of methylene chains is still

uncertain, while below the Tc the methylene chains become stiff and lie parallel to each other and perpendicular to the plane of the membrane. In the case of biological membranes, phase changes due to temperature alteration are also frequently reported but again are poorly understood. The presence of numerous lipid species in a biological membrane introduces the possibility of multiple phase transitions and/or separations. Thus, any discussion dealing with thermal effects necessitates caution. In the case of the $(Na^+ + K^+)$-ATPase, the thermally induced phenomenon is definite but whether it is a consequence of a phase separation or transition or both is not known. Three points have been well substantiated:

i) A change in the membrane occurs on cooling.

ii) This change usually occurs at about 20°C and is dependent on lipid(s).

iii) The temperature induced lipid change alters the function of the enzyme protein, resulting in a higher activation energy for ATP hydrolysis.

b) By comparison with the activation experiments, lipid analysis of $(Na^+ + K^+)$-ATPase preparations would appear to be a relatively unpopular approach towards the understanding of the involvement of phospholipid in the mechanism of this enzyme. This approach is based on the logic that since purification leads to a concentration of the protein moiety any closely bound lipid would also be concentrated. The most exhaustive study in this area is the work by Wheeler and his coworkers (Wheeler *et al.*, 1975; Wheeler and Walker, 1975; Wheeler, 1975). They studied two methods of enzyme purification, namely DOC-treatment and a sequential NaI-Lubrol extraction. With both preparations they

showed a marked correlation between specific activity and the phospholipid content, when expressed per unit protein. In an examination of the nature of the phospholipids they did not detect a selective loss or concentration of any particular kind of phospholipid in either the DOC-treated membranes or the Lubrol-treated membranes. It is noteworthy that an untreated preparation and a DOC-treated preparation differed slightly in phospholipid composition even though the latter was fifty-three times as active. The more pure preparation showed a small decrease in PC and some increase in lysophosphatides. Wheeler and his coworkers have suggested that although phospholipid specificity by headgroup could not be excluded as a requirement for ATP hydrolysis, additional specificity by fatty acyl chain might exist. In a separate communication (Walker and Wheeler, 1975) circumstantial evidence for this view was provided by an examination of the fatty acyl chains of two phospholipids (PC and PS) which are known to differ markedly in their potential for reactivation (Palatini *et al.*, 1972; Kimelberg, 1976). PS, the more active of the two had 15% C-20 and 83% C-18 chains while PC contained no C-20, 65% C=18 and 35% C-16 chains.

This work supports the suggestion made earlier by Tanaka and Teruya (1973). Their analysis of the phospholipid fatty acyl chains of $(Na^+ + K^+)$-ATPase preparations from the brains of the bull frog and the ox showed a preponderance of unsaturated long chains of 18 or 20 carbon atoms. They concluded that the physical state of the enzyme was a decisive factor, and that its conformational changes might be dictated by an activating lipid ligand.

More recently Palatini *et al* (1977) concluded from their work that a requirement for fluid chains and negatively charged polar head

groups existed to "assemble in an expanded lamellar configuration".

Thus it is apparent that the analytical approach towards the elucidation of the lipid involvement is also incomplete. Phospholipids having acidic head groups and long unsaturated fatty acyl chains are most suited to the reactivation of delipidated ($Na^+ + K^+$)-ATPase preparations. At the other extreme however, PS and cholesterol ester have been shown to increase with purification of the ($Na^+ + K^+$)-ATPase (Kawai *et al*, 1973) and sulfatide and enzyme content have been shown to increase in parallel in the salt gland of ducks that had been fed a high salt diet (Karlsson *et al.*, 1971).

c) Studies of lipid interactions in the partial reactions have also made use of delipidation procedures. In 1971 Taniguchi and Tonomura treated ox brain microsomes with bee venom PPL-A and studied the enzyme-phosphate intermediate. They suggested that since the formation and the breakdown of this complex were both reduced by treatment with PPL-A, therefore these two stages required some lipid. Since PPL-A does not directly act on the protein moiety of the ($Na^+ + K^+$)-ATPase, it is implied that the contribution of the lipid is towards maintaining a particular conformation(s). Goldman and Albers (1973) using membranes from Electrophorus electroplax found data to support the observation made by Taniguchi and Tonomura (1971). Furthermore they also suggested that the conformational change from E_1-P to E_2 - P as well as the final step, the E_2 to E_1 conversion were both lipid dependent. This concept of an all embracing effect of the lipid is not shared by Barnett and Palazzotto (1974). Focussing their attention on the effects of temperature, they showed that neither the terminal catalytic step, the so

called K^+-pNPPase nor the binding of [^{3}H]-ouabain to these preparations showed any discontinuity in the Arrhenius plot. Therefore, the authors conclude, these two partial reactions are not lipid dependent. Using both approximations and presumptions, they developed an argument which "leaves the conversion of E_2-K to E_1 as the most likely site of action of the phase transitions". Because of the weakness of their argument it is not pertinent to compare the suggestion made by Barnett and Palazzotto (1974) with the works cited earlier (Taniguchi and Tonomura, 1971; Goldman and Albers, 1973); there is however one advantage to the approach taken by Barnett and Palazzotto namely, that it permits the study of temperature effects on untreated membranes.

[1]Charnock *et al.*, (1975) used an approach which compared Arrhenius plots of specific activity obtained by ouabain inhibition of the enzyme with those obtained by cation activation. Rabbit kidney membranes subjected to six different treatments were examined: DOC, nonidet P-40, sodium iodide, phospholipase-C, and phospholipase-A alone and after addition of phosphatidyl serine. Our studies showed that above Tc the Ea_1 did not differ appreciably whether obtained by cation activation or by ouabain inhibition. Below Tc however there was a measurable difference. For example with the untreated membranes Ea_2 from cation activation data was 60% of the Ea_2 from ouabain inhibition data. This trend was most marked in data obtained from membranes treated with sodium iodide, a chaotropic agent which disrupts lipid-protein and protein-protein interactions in biological membranes.

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[1]Charnock, J.S., Almeida, A.F., and Rebecca To. (1975) Arch. Biochem. Biophys. 167. 480-487. This work will not appear either in the methods section or in the results section. For completion, a copy is included in the appendix.

(Hamaguchi and Geiduschek, 1962; Hatefi and Haustein, 1969). The results obtained from PPL-A treated membranes was also noteworthy. With cation activation the discontinuity of the Arrhenius plot was retained. This is in contrast to the ouabain inhibition data as was also found previously (Charnock *et al.*, 1973). In general, all the treatments indicated that cation activation of the enzyme was less influenced than ouabain inhibition. The suggestion was made that the phase transition of the lipids influences the $(Na^+ + K^+)$-ATPase enzyme protein, and this effect was much more apparent when ouabain inhibition was examined rather than cation activation of the enzyme. We made a further suggestion from the unique effects of PPL-A on the membrane. Since the apparent lysis of PS affected ouabain inhibition and not cation activation, the possibility now existed that this phospholipid generally or in a specific location imparted cardiac glycoside sensitivity to the enzyme. This latter suggestion contrasts with the claim by Barnett and Palazzotto (1974) that ouabain inhibition of the enzyme yielded a linear Arrhenius plot.

Wheeler's group (1975) studied the partial reactions of this enzyme using rabbit kidney membranes that had first been treated with NaI and then partially delipidated with Lubrol-W. They found that Na-dependent phosphorylation was partially inhibited by delipidation but K-dependent dephosphorylation was totally inhibited. They offer the interpretation that the partial reactions up to and including E_1 - P formation are either not dependent on lipid, or dependent on tightly bound lipid. By contrast phospholipid was essential for the transformation of the E_1 - P to E_2 - P and subsequent dephosphorylation. Thus this latter work finds common ground with all of the four works dis-

cussed before it. From the literature then it appears that the E_2 - P conformer through its formation and/or breakdown is the pivotal point that links the lipid-protein interactions, the conformation changes and the thermal phenomenon of this enzyme. In this regard, Rivas *et al.* (1972) showed that when the tritiated drug-enzyme complex E_2 - P - O was extracted with chloroform-methanol, the organic phase contained a hydrophobic protein that carried the ^{3}H-ouabain. Because of the denaturing effects of organic solvents however, this work is usually received with more caution than attention.

d) In recent years some workers (Goldin and Tong, 1974; Hilden *et al.*, 1974; Goldin and Sweadner, 1975; Hilden and Hokin, 1976; Anner *et al.*, 1977) have succeeded in introducing lipid depleted highly purified ($Na^+ + K^+$)-ATPase proteins into homogeneous liposomes of known phospholipid composition. The ($Na^+ + K^+$)-ATPase-loaded liposomes show coupled catalytic and cation pumping activity and also display sensitivity to ouabain. There is some question as to the efficiency of pumping and to the ion specificity. For example, Goldin and Tong (1974) (Goldin and Sweadner, 1975) observed that the pumping of Na^+ was accompanied by chloride pumping which they suggested was unique characteristic of kidney ($Na^+ + K^+$)-ATPases. Anner *et al* (1977) however could not support this view with their data. They suggested that the method of purification could alter the final outcome and that renal ($Na^+ + K^+$)-ATPase as compared with the enzyme from other organs, might be more susceptible to the manipulations administered during the process of purification.

Employing the phospholipid exchange technique of Warren *et al.*, (1974a,b), Hilden and Hokin (1976) were able to obtain a ($Na^+ + K^+$)-ATPase

preparation in a pure phosphatidyl choline matrix, showing that their enzyme preparation from dogfish shark rectal salt gland can function in the absence of other phospholipids. They also claimed that Na^+ and K^+ pumping was now twice as effective as was observed with endogeneous phospholipids. However this cannot be taken at face value for at least one important reason. The optimal activity of an enzyme preparation necessitates the determination of an optimal temperature of operation. In lieu of the involvement of lipids in the temperature sensitivity of this enzyme, any alteration of the lipid species can only make such comparisons of activity approximate at the best.

Although such reconstitution experiments will have a place in the investigative history of the pump-enzyme, they have contributed very little towards an understanding of its mechanism of action or its interaction with the membrane phospholipids. The following points stand out:

i) The difficulty of orientation of this macromolecule within the bilayer persists. This problem has special significance for a macromolecule with the defined vectorial properties of the ($Na^+ + K^+$)-ATPase. It is possible that orientation of the macromolecule also has a requirement for lipid.

ii) The sodium to potassium pumping ratio of 3:2 has yet to be established conclusively. This stoichiometry has special significance for a pump that must maintain a transmembrane electrochemical gradient.

iii) The optimum temperature of the pumping mechanism for the Hokin reconstituted enzyme was 25°C (Hilden and Hokin, 1976). This may reflect a suboptimal lipid-protein interaction.

iv) Finally it is a telling point that the reconstitutions which include five different enzyme sources (dog and lamb kidney, beef heart and brain, and Squalus acanthias rectal gland) have all been achieved with phosphatidyl choline, and not with phosphatidyl serine, a lipid known to be more effective in reactivation studies (Palatini *et al.*, 1972; Kimelberg, 1976). Presumably this is not for want of trying but from failure to obtain this result.

Perhaps in the biological sense synthetic rather than reconstituted pump-enzyme would be a more appropriate definition. However, semantic improprieties must not stand in the way of the credit that is due to the workers in this field. They have conclusively demonstrated an extremely important point which has concerned the critics of this enzyme from the days of its first description by Skou in 1957. There can no longer be any doubt that the enzyme macromolecule is indeed the 'Sodium Pump' of mammalian cells.

Rationale

From the introduction it is apparent that neither the clinical nor the biochemical pharmacology of the cardiac glycosides can as yet be adequately explained at the molecular level. However the working hypothesis that the pump-enzyme $(Na^+ + K^+)$-ATPase is itself the drug-receptor, provides the basis for direct experimental investigation.

This project utilizes an *in vitro* approach to study the $(Na^+ + K^+)$-ATPase enzyme at the molecular level.

The rationale for this particular work is based on two facts. First, phospholipid is essential for the catalytic function of the en-

zyme, and second, catalytic function is uniquely sensitive to temperature. From this it can be argued that a change in the catalytic function of enzyme enriched membranes, as for example after treatment with detergents or lipases, may be due to qualitative and/or quantitative alteration of the phospholipids. One method of study of this interaction is the Arrhenius analysis of the temperature-activity relationship of the system. For example, after treatment with detergents or lipases, an overall change in membrane fluidity as it affects enzyme activity may be detected. Thus these experiments should permit us to obtain a better understanding of the effects of membrane fluidity on enzyme function and enzyme inhibition by cardiac glycosides.

This will be achieved by first exposing the enzyme enriched membranes to treatments that are known to effect the specific activity of $(Na^+ + K^+)$-ATPase, followed by an examination of the effects of temperature on the three parameters listed below:

1) The catalytic activity (ATP hydrolysis).
2) The binding of ouabain to the system.
3) The fluidity of the membrane matrix as reported by an ESR probe intercalated in the bilayer.

METHODS

1. General

Distilled water, passed through a battery of deionizing columns was used to prepare all aqueous solutions. The reagents were of Analar grade or better, depending on what was available. The suppliers have been listed in an Appendix III. After the solutions had been made to volume, they were filtered through a millipore filter of pore size 0.8μ.

2. Enzyme preparation

2.1 Solutions

Homogenizing buffer contained 250mM sucrose, 30mM L-Histidine (base), 5mM H_4-EDTA adjusted to pH 6.8 with 1000mM Tris-base.

Wash buffer contained 20mM Tris-base and 1mM H_4-EDTA brought to pH 7.6 by the addition of 1000mM HCl.

Sucrose buffer contained 275mM sucrose, 0.20mM H_4-EDTA, and was brought to pH 7.6 with Tris-base.

2.2 Enzyme preparation

Ox brain (bos taurus) was obtained fresh from the abattoir and chilled in crushed ice while it was still warm. The cortex was cleared of large blood clots and rapidly frozen in liquid nitrogen, in 200g portions for storage in a freezer at -20°C. (Na^+ & K^+)-ATPase enzyme preparations were made from frozen or fresh ox brain, according to the general procedure of Charnock and Post (1963).

The frozen tissue was left at room temperature to warm but was not allowed to thaw. While still frozen it was cut into small pieces of about 1g and then allowed to thaw in homogenizing buffer at 0°C.

The thawed tissue, in a ratio of not less than 1g tissue to 10ml buffer was homogenized. Homogenization was carried out at 4°C using either (a) four strokes of a teflon-glass homogenizer, or (b) one 10 s pulse of a Polytron Homogenizer (Brinkmann Instruments, Canada Ltd.) fitted with a PT-20 generator and operated at setting 8. All subsequent steps were performed at 4°C.

Cellular debris was removed by centrifugation at 1000 x g for 15 min in a refrigerated Sorvall RC2-B centrifuge fitted with a SS34 rotor. Further centrifugations were performed at 9000 x g for 20 min to remove possible contamination from mitochondrial particles. A heavy microsomal pellet was isolated from the 9000 x g supernatant by centrifugation at 46000 x g for 30 min, then washed twice by resuspension in wash buffer, and centrifugation again at 46000 x g. The washed pellets were resuspended in sucrose buffer, and stored at -20°C after rapid freezing in liquid nitrogen. The protein content of these microsomal suspensions was determined by the method of Lowry *et al.*, (1951) as described below. Protein concentrations ranged from 2 to 8 mg/ml.

2.3 Treatment of the enzyme with deoxycholic acid (DOC)

Treatment with this detergent was carried out by mixing equal volumes of ($Na^+ + K^+$)-ATPase enzyme suspension (1mg/ml wash buffer) and DOC (2mg/ml wash buffer). After allowing the reaction to proceed for the appropriate time, it was stopped by the addition of an equal volume of ice cold wash buffer. The DOC-treated membranes were recovered by sedimentation at 46000 x g for 30 min. This residue was washed and stored in a manner identical to that described for the untreated membranes. Three different DOC treatments were employed:

i) 0.1% DOC for 10 min at 4°C.

ii) 0.1% DOC in the presence of 3mM ATP for 30 min at 30°C.

iii) 0.05% DOC in the presence of 2mM ATP, 5mM $MgSO_4$ and 80mM NaCl for 5 min at 30°C.

Results from preliminary experiments (see Appendix IV) showed that method (ii) was the most appropriate, and it was used routinely thereafter. Unless stated otherwise all DOC-treated membranes were purified by exposure to 0.1% DOC in the presence of 3mM ATP for 30 min at 30°C.

In a separate experiment [Carboxyl-^{14}C]-DOC was used to estimate the residual detergent in an enzyme preparation. The sodium salt of DOC was supplied as 150 µl of an aqueous solution containing 2% ethanol (Amersham/Searle). The solution contained 9.62×10^{-4} µmoles ^{14}C-DOC with a specific activity of 52 µCi/µmole. The radiochemical was made up to 104 ml with 0.2% DOC pH 7.6. Thus the working solution contained 0.2004% DOC and 0.003% ethanol and had a specific activity of 1.1×10^6 dpm/ml.

In all experiments where ATP was used in the purification process, the membranes were first preincubated at the required temperature along with the ATP for a few minutes to permit binding of the nucleotide to the enzyme, following which the DOC was added.

2.4 Treatment of the enzyme with sodium dodecyl sulfate (SDS)

Treatment with SDS was carried out by mixing equal volumes of ($Na^+ + K^+$)-ATPase enzyme suspension, 1 mg/ml in wash buffer containing 8 µmoles ATP per ml with 0.1% SDS at 30°C. After allowing the reaction to proceed for the appropriate time, it was stopped by the addition of an equal volume of ice cold wash buffer. The SDS-treated membranes

were recovered by sedimentation at 46000 x g for 30 min. This residue was washed and stored in a manner identical to that described for the untreated membranes. From the preliminary experiments (see Appendix V), 30 min exposure to 0.05% SDS in the presence of 4mM ATP at 30°C was chosen as a routine purification procedure thereafter.

In a separate experiment SD^{35}S was used to determine the residual detergent in the enzyme preparation. Lauryl sulfate, sodium salt [^{35}S] was supplied as a dry powder (New England Nuclear). It was dissolved in 4 ml distilled water and 1.0 ml of this stock solution together with 0.8 ml 5% SDS were made upto 50 ml with Tris-EDTA buffer to give a 0.1% solution of SD^{35}S with a specific activity of 5.55×10^6 dpm/ml.

2.5 Treatment of the enzyme with phospholipase-A (PPL-A) and reactivation with phosphatidyl serine (PS).

The procedure for treatment of the particulate enzyme with PPL-A and subsequent reactivation with PS was adapted from that of Imai and Sato (1960). The $(Na^+ + K^+)$-ATPase containing membranes, 1 mg protein/ml were warmed to 37°C in a medium buffered to pH 7.4 containing sucrose, 275mM, serum albumin 7.35 mg/ml, Tris-base, 14.7mM, 2-mercaptoethanol 3.37mM, EDTA, 0.035mM, and $CaCl_2$, 5.6mM. The reaction was started by the addition of bee venom PPL-A (Sigma Chemical Co.) 1180 units per mg $(Na^+ + K^+)$-ATPase protein, and allowed to continue for 12 min. The reaction was terminated by the addition of 0.67 volumes EDTA 57mM-Tris 321mM, followed by immediate dilution to 3 volumes with ice cold distilled water. The treated membranes were sedimented at 77,500 x g for 3 hr in a Beckman L-3 ultracentrifuge Type 60TI. The pellets

were washed and resuspended as described for untreated membrane.

Reactivation after PPL-A treatment was achieved by incubating equal volumes of PPL-A-treated membranes 1 mg/ml and PS liposomes, at 37° for 10 min. The liposomes were prepared by sonicating 3 mg PS per ml sucrose buffer in a Cole-Parmer Ultrasonic cleaner Model 884S-4 at about 35° till the solution was clear.

2.6 Organic solvent extraction and cholesterol determination

Membrane aliquots were diluted 10-fold with distilled water and sonicated for 1 min in a Cole-Parmer Ultrasonic Cleaner, Model 884S-4 and then sedimented at 48000 x g for 10 min in a Sorvall RC 2B centrifuge. The supernatants were assayed for protein content. The pellets were transferred into weighed test tubes with MeOH, and $CHCl_3$ was added to obtain a 1:2 ratio of the respective organic solvents. The mixture was again sonicated for 1 min and then centrifuged in a bench top centrifuge, model 8845-4. The clear supernatant was poured off and the pellets re-extracted twice more in the same way. The collected supernatants were evaporated in a stream of air and the residual lipid dried under vacuum in a dessicator containing silica. This lipid extract was redissolved in $CHCl_3$ and stored at -20° until it was used.

The cholesterol content of the lipid extracts was determined by a modified Libermann-Burchard reaction (Huang *et al.*, 1961). Aliquots of the organic solvent extract were evaporated to dryness in glass tubes equipped with teflon lined screw caps. The residue was shaken with 100 µl glacial acetic acid and warmed in a water bath to 25°. The colour reagent composed of 150 ml acetic acid, 300 ml acetic anhydride, 50 ml sulfuric acid (96%), and 10 g anhydrous Na_2SO_4, 5 ml per tube. The colour was allowed to develop for 25-30 min and the

absorbance at a wavelength of 632nm was determined in a Beckman DBG spectrophotometer. The optical density was read against a standard curve derived with cholesterol dissolved in acetic acid.

2.7 Determination of enzyme specific activity and enzyme protein concentration

All enzyme preparations were routinely assayed for both ouabain sensitive and ouabain insensitive ATPase activity at 37°. In some instances, in particular when the effects of temperature on [^{3}H]-ouabain binding were studied, a full temperature profile of ATPase was also constructed.

Enzyme activity was measured by the coupled optical assay system which continuously monitors the oxidation of NADH at 340 nm (Schoner *et al.*, 1967; Schwartz *et al.*, 1969). A Gilford 2400 recording spectrophotometer equipped with a jacketed ethylene glycol/water constant temperature bath was used in these experiments. The temperature of the spectrophotometer cell was controlled at $\pm$ 0.2°C. Reactions were performed in 100mM glycylglycine (pH 7.6) containing 2mM Mg SO_4, 80mM NaCl, 20mM KCl and 250mM sucrose which was added to prevent protein settling. The assay ingredients included 3.14mM phosphoenol pyruvate (sodium salt) and 64 units of pyruvate kinase, 19 units lactic dehydrogenase and 0.2 mg NADH in a final volume of 3 ml. The membrane enzyme preparation was added to a temperature equilibrated cuvette and allowed to equilibrate for a further minute before the reaction was initiated by the addition of ATP to a final concentration of 1.5 mM. Monitoring of NADH oxidation commenced immediately after mixing of the cuvette contents with a small plastic swizzel stick. Mg^{++}-ATPase activity was followed in the pre-

sence of 0.4mM ouabain, and was subtracted from the total ATPase activity obtained in the presence of Mg, Na, and K to give the ouabain sensitive ($Na^+ + K^+$)-ATPase activity.

For the estimation of the ($Na^+ + K^+$)-ATPase protein by the Lowry assay (Lowry *et al.*, 1951), a standard curve of not less than 5 points was constructed for each protein assay using bovine serum albumin. The absorbance of the samples was read at 650 nm on a Beckman DB-G spectrophotometer or a Gilford Micro Sample spectrophotometer Model 300N.

2.8 Arrhenius plots

The Arrhenius equation provides a means whereby the energy of activation of a reaction can be determined. In graphic form (log K versus 1/T °K), the Arrhenius plot illustrates the sensitivity of the reaction to temperature. The slope of the line derived from such a plot provides the energy of activation of the reaction.

A number of biological reactions do not conform to the Arrhenius equation and generate a non-linear plot between 37°C and 5°C. The most serious problem in the application of Arrhenius plots to biological systems, is a determination of the number of discontinuities. During the early work in this laboratory (Charnock *et al.*, 1971), considerable importance was attached to the possibility that a visual assessment might introduce bias against a linear slope. Consequently a technique of Bogartz (1968) was employed to fit two lines to a cloud of points. The point of intersection of the two lines was obtained and defined as the "point of inflection" or Tc. Furthermore an analysis of variance was applied to the two lines above and below the Tc to ensure that they did not deviate significantly from linearity. These

operations were incorporated in a computer programme that would display the data graphically, as well as calculate the value for Tc and the values for the two activation energies above and below this inflection (Cook and Charnock, in press). For the correct application of this programme, it is essential that the readings for each replicate be obtained at the same temperature point (i.e. constant values for the abscissa) and that some numerical manipulation can be applied to similarly standardize the values for the ordinate. The latter was achieved by setting the value for the rate obtained at 37°C as 100% and expressing all subsequent rates obtained at lower temperatures in these terms.

This method was thus most appropriate for deriving the parameters described above in those experimental protocols where a differentiation between linear and non-linear plots was of prime importance. The method has not yet been developed to the level where it can be applied under circumstances where more than one inflection point might exist.

A part of the results presented in this work includes results obtained from ESR experiments. These data could not at first be achieved at fixed temperature points. In order to standardize these data, the individual points were first joined by straight lines. From these lines readings for the ordinate were determined at fixed temperatures for each replicate which were then averaged and statistically evaluated. These averaged data were then subjected to the Arrhenius form of presentation, using the computer programme described above.

Subsequent use of a newer and more precisely temperature controlled ESR spectrometer made it possible to obtain data at 1°C inter-

vals from 37°C to 1°C. These data could not however be subjected to the numerical standardization described above for the hydrolytic activity (i.e. as a % of the value at 37°C which was set at 100%), because the distribution of standard errors was non-uniform. A suitable method was not found whereby the replicate data could be accumulated and subjected to statistical analysis. All the methods that were attempted were found to bias the results in favour of or against some aspect of the data. A more detailed discussion of this problem appears in the chapter on discussion. In order to avoid these problems, the approach used by Ashcroft *et al.* (1977), namely a simple plot of mean values with the ranges versus some ESR parameter has been adopted.

3. Ouabain binding

3.1 Solutions

The wash solution contained 100mM glycylglycine, 2mM $MgSO_4$, 80mM NaCl, 0.2mM EDTA, and was adjusted to pH 7.6 with Tris-base. It also contained a concentration of unlabelled ouabain equal to that in the binding medium.

The binding solution consisted of a wash solution containing 2mM ATP, pH 7.6. The binding solution also contained [^{3}H]-ouabain from New England Nuclear at the desired concentration (usually 5 x 10^{-7}M) with the specific radioactivity maintained between 300 - 400 dpm/pmole ouabain where possible.

3.2 Ouabain binding

The binding reaction was carried out in a jacketed glass vessel of about 2.5 cm internal diameter, connected to a constant temperature ethylene glycol/water bath. The reaction was initiated at time 0

by the addition of 0.1 ml ($Na^+ + K^+$)-ATPase (100 to 300 µg protein) to 2.4 ml binding solution pre-equilibrated for 5 min to the desired temperature. A magnetic stirrer was used to maintain constant mixing.

A plastic box was constructed to hold six millipore filter funnels equipped with scinter beds. The filter funnels and millipore filters 0.8µ pore size, were pre-washed just prior to initiation of the binding reaction with 2 x 2 ml wash solution (maintained at the temperature of the reaction) delivered by an automatic syringe. The pre-wash eliminated [^{3}H]-ouabain binding to the filters and also maintained a constant suction pressure on all the filters. Binding times were taken to the time when 1 ml aliquots of the reaction medium were applied to the filters. At 37° aliquots were taken at 5 s intervals. At the lower temperatures these sampling intervals were increased so that at 9° the whole operation took 250 s. However at all temperatures samples were removed at much longer intervals, up to 60 min at 9°, so that both rates and equilibrium levels of [^{3}H]-ouabain could be obtained.

Rates of [^{3}H]-ouabain binding were determined from the slopes of a regression analysis of the levels of [^{3}H]-ouabain bound, using the Olivetti program #681009. Assays were always in duplicate and in some cases in triplicate or more. Disagreement between individual assays was always less than $\pm$ 5%, and the mean values obtained were very reproducible upon repeated assay of samples stored at -20° for several days. No correction was made for any residual non-specific binding of [^{3}H]-ouabain to protein, as in the absence of activating ligands, this was found to be negligible in these experiments.

3.3 Radiolabelled ouabain and counting procedures

Tritiated ouabain from New England Nuclear was supplied as a

solution in ethanol with a specific activity of 12 Ci/mM. The ethanol was blown off in a gentle stream of nitrogen and distilled water added to give a stock [^{3}H]-ouabain solution of about 10 pmoles ouabain per ml, and a specific activity of about 1 x 10^9 dpm per ml.

After filtration of the enzyme protein the millipore filters were dried in air, disintegrated in 1 ml MeOH and counted in a fluor of the following composition: Naphthalene 100 g, PPO 5 g, Dioxane 720 ml, and Toluene 142 ml. The samples were counted to 3% error in a Beckman LS-100 liquid scintillator with a 33-38% efficiency.

4. Electron spin resonance

4.1 General comments

Electron spin resonance (ESR) has been used for a number of years to study biological processes. Until 1965 the application of this technique was confined to biochemical reactions involving two phenomena:

a) the formation of free radicals as for example during enzyme reactions, carcinogenesis or radiation damage, and

b) the presence of paramagnetic metals as for example in metalloenzymes.

In 1965 Ohnishi and McConnell and Stone *et al.*, introduced the stable free radical and as a consequence the application of ESR to biological investigations expanded rapidly. The extent of this sudden interest is apparent from the many reviews and books that have appeared.

The free radical is usually a nitroxyl group stabilized by incorporation into a ring structure to produce a spin label. The spin

label can be linked to a variety of biological molecules that may be substrate, drug or other molecules, to form spin probes. The spin probes are relatively stable in the normal biological temperature range and in the pH range 3 to 10.

An unpaired electron possesses a spin which gives rise to a magnetic dipole. According to the quantum theory the spin angular momentum of electrons can only occur in integral multiples of a quantum and therefore the magnetic moment associated with the electron must also be quantized. Consequently an unpaired electron when placed in an external magnetic field can assume only two possible orientations; their magnetic dipoles will be aligned either parallel or antiparallel to the external field. The splitting of the allowed energy states of the electron in an applied magnetic field is called the electronic Zeeman effect. More electrons will be in the lower energy state (parallel to the external magnetic field) and the technique of ESR is to induce spin transitions from the lower level to the higher level by the application of electromagnetic radiation (microwaves). The electrons in the upper energy state lose energy through relaxation processes and fall to the lower level. In this way net absorption of energy is maintained.

An unpaired electron in a free radical or spin label molecule will interact with other magnetic dipoles present in the molecule. Since some nuclei within the molecule may possess nuclear spin and hence magnetic dipoles, these nuclei can interact with the unpaired electron and with the applied magnetic field. The orientation of a nuclear spin I with the magnetic field is quantized into $2I + 1$ allowed orientations. In each of these nuclear spin states, the interaction

between the electronic and nuclear magnetic moments is different. The interaction of an unpaired electron with a nucleus with spin I therefore gives rise to a hyperfine splitting of the ESR spectrum into 2I + 1 transitions, each of which can be labelled by the nuclear spin quantum number M_I. In a nitroxyl spin probe, the unpaired electron is localized in the region of the N-O fragment and experiences a hyperfine interaction with the ^{14}N nucleus (spin I = 1) so that the isotropic ESR spectrum consists of three lines whose separations are characterized by the hyperfine splitting constant A_o (or a_o). The field at the centre of the ESR spectrum is a measure of the g-value (spectroscopic splitting) of the electron in its molecular environment. For nitroxides, g = 2.006.

A typical isotropic ESR spectrum for a nitroxide is shown in Fig. 1, where the first derivative of the absorption is plotted against the applied magnetic field. The figure also indicates some spectral parameters and their corresponding symbols. Thus, h_{+1}, h_o and h_{-1} denote the peak heights of the low-, the mid- and the high-field lines while the widths of these lines are denoted by W_{+1}, W_o and W_{-1} respectively. The subscripts +1, 0 and -1 refer to the allowed values of M_I, the nitrogen spin quantum number.

The spectrum shown in Fig. 1 is characteristic of rapidly rotating spin labels. Using some approximations, theories of line widths have been developed for short correlation times (Kivelson, 1960; Freed and Fraenkel, 1963). From these theories an expression has been derived (Raison *et al.*, 1971) which relates the rotational correlation time τ_o to measurable peak heights and widths.

Thus: $$\tau_o = K W_o [(h_o/h_{-1})^{\frac{1}{2}} - 1] \text{ s}$$

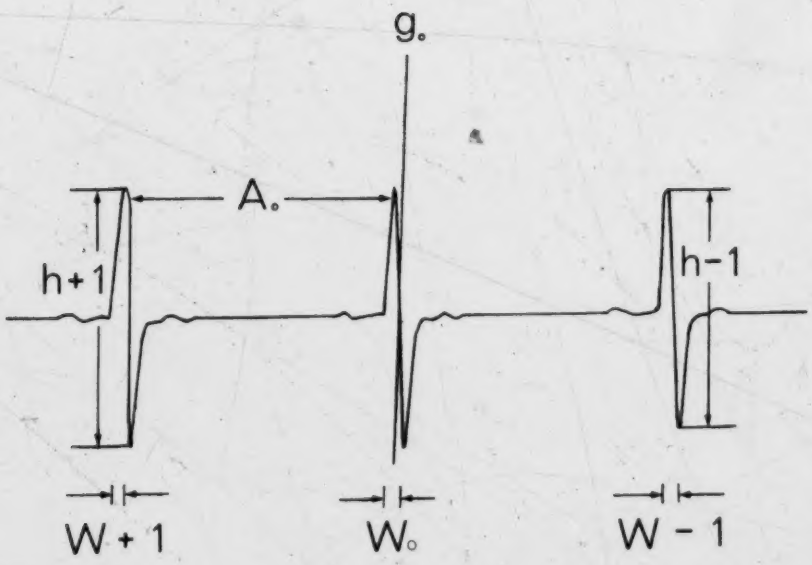


Fig. 1.

where: K is a constant = 6.5×10^{-10} s for nitroxides as determined from the spectral data of Griffith *et al.* (1965).

W_o is the line width of the centre line and h_o and h_{-1} are the peak heights of the mid- and low-field lines (see Fig. 1).

This formula although it incorporates some assumptions and a number of approximations, gives a reasonable measure of the correlation time for freely tumbling spin labels in an isotropic environment.

When nitroxides are restricted in their motion, spectral anisotropy is observed, and the spectroscopic splitting factor g as well as the splitting constant A are now both angular dependent. By incorporating spin labels in crystals, the values of the hyperfine splitting and g-value can be determined (Libertini and Griffith, 1970; Seelig, 1970) when the field is parallel to or perpendicular to the N-O axis of the molecule. These parameters have been illustrated in sub-section 4.7.

4.2 Instrumentation

Some of the ESR spectra were obtained using a Varian V-4502 BST spectrometer. This instrument is equipped with an Alpha Model 3039 digital NMR gaussmeter for magnetic field calibration and a Varian-4557 temperature control. The temperature of the sample chamber was examined by a copper-constantin thermocouple to 0.1°. Microwave frequencies were monitored with a HPX 532B frequency meter. In addition, some spectra were obtained using a Bruker B-ER 420 spectrometer coupled to a Varian Magnet VFR-240B. This spectrometer is equipped with a Bruker temperature control unit Model #-ST 100/700, which employs a chrome-alumel thermocouple. This latter unit maintained the temperature of the sample to 0.1° and also permitted the very detailed temperature studies.

4.3 Sample tubes

Although quartz glass cells have traditionally been used for esr spectrometry, the application of this physical technique to biological investigation has introduced the use of an assortment of glass capillary tubes including pasteur pipettes, micropipettes, and hematocrit tubes. The obvious advantage of this improvisation is the low cost of these sample tubes which obviates the need for cleansing and reuse. A disadvantage however, is the presence of paramagnetic compounds or metals in the glass. This paramagnetic contamination was observed in the four varieties of glass tube examined, and these are listed below.

1) Microhematocrit capillary tubes (Blue-Tips, Plain. Fisher Scientific).
2) Microhematocrit capillary tubes (Capilets - Dade. Canlab.).
3) Micropipettes (Microcaps. Drummond.).
4) Pasteur pipettes (Dispopipets. Fisher Scientific).

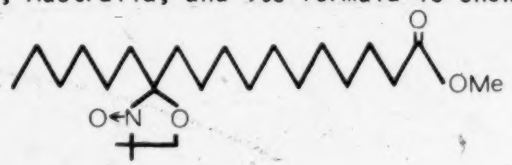
The variation in the paramagnetism of these four tubes was not very marked; the microhematocrit tubes from Fisher Scientific, were chosen as they were readily available. By restricting the gain as well as the modulation amplitude, it was possible to restrict the intensity of the contaminant peak to an acceptable level.

The sample tubes were first sealed at one end after which the label was introduced as a solution in methanol. The solvent was blown off in a stream of nitrogen and the label remained as a film on the surface of the glass tube. Liposome or microsome suspensions were then placed in this tube and incubated at 37° for 10 min to permit

diffusion of the spin label from the glass.

4.4 The spin label

The spin label, N-oxyl-4' 4'-dimethyl oxazolidine-12 keto methyl stearate (M 12-NSE) was a gift from Dr. J.K. Raison, Macquarie University, Sydney, Australia, and its formula is shown below:



The main advantage of this label was that at the time this project was commenced, spin probes of membrane lipids were not commercially available. In addition it had also been tried on biological membranes and found to report from within the lipid bilayer (Raison *et al.*, 1971).

A serious problem in the application of ESR spectroscopy to the examination of biomembranes is the possibility of line broadening due to interactions between the electrons of the label molecules when pooling or clustering of the label occurs (Scandella *et al.*, 1972). In order to avoid this spin-spin coupling effect it was essential to establish a concentration of label that could be employed within the limits of the gain and modulation amplitude that were referred to above. Fig. 2 gives the results of experiments designed to investigate these limits. The contribution from the glass is shown in Fig. 2a and the gain settings on the spectrometer were restricted such that this amount would not be exceeded. The spectrum of 2 nmoles M 12-NSE as a film on the walls of the sample tube is shown in Fig. 2b. This spectrum which was obtained at a similar

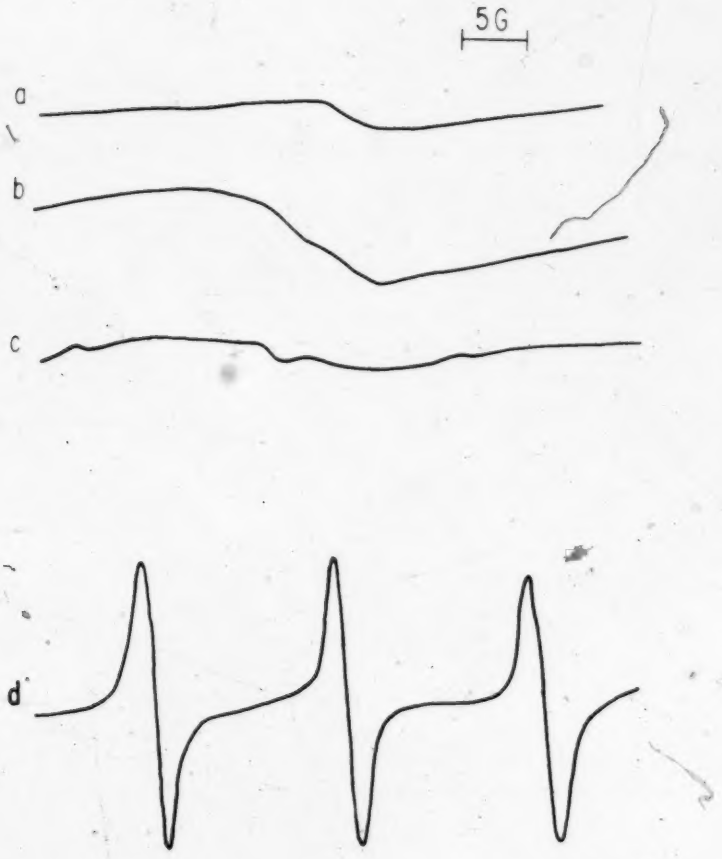


Fig. 2 Limits of detection. The ESR spectra shown were obtained from (a) the empty sample tube; (b) 2 nmoles of M 12-NSE as a film on the surface of the empty sample tube; (c) after the addition of buffered sucrose solution to (b) above; (d) from 0.5 nmoles M 12-NSE in methanol.

gain setting to Fig. 2a shows a broad signal superimposed on the signal recorded from the glass. Together they appear as a distortion of the base line. However, this spectral contribution will be reduced when some of the label becomes incorporated into the membrane. Thus, the base line distortion due to label on the walls of the sample vessel must be less than that shown in Fig. 2b. Fig. 2c shows an examination of the effects of a buffered sucrose solution on this label film. Immediately after the addition of the aqueous phase of the system no change could be detected in the spectrum. However, after 30 min contact, peaks of low intensity were apparent which were superimposed on the broad spectrum of the film. Subsequent more prolonged exposure of up to 24 h did not alter the intensity or shape of these peaks. At this concentration of probe there is little solubilization of the M 12-NSE in the buffered sucrose medium. By comparison when a solution of M 12-NSE in methanol was examined at the same gain settings as those used above, a typical spectrum was obtained but the intensity of the peaks was so great that the concentration of the label had to be reduced four-fold (0.5 nmoles/25 μl) to accommodate in the Fig. 2b.

From these experiments four important points can be made:

1) The contribution from label in aqueous solution is negligible.

2) Excess label present as a film on the surface of the sample tube does not make a significant contribution to the spectrum.

3) A concentration of 2 nmoles M 12-NSE represents a four-fold excess of label that can be detected at the maximum gain settings to be used.

4) The paramagnetic signal in the glass sample tube has been contained.

4.5 DMPC liposomes as control bilayers

It is well known that liposomes made from synthetic phospholipids will incorporate nitroxide spin labels (Butler *et al.*, 1974). In liposomes labelled with fatty acid spin labels, the long axis of the spin probe is oriented perpendicular to the plane of the bilayer (Seelig 1970; Hubbell and McConnell, 1971). Thus the line shape of the spectrum will vary depending on the depth to which the nitroxide reporter group of the label is embedded in the bilayer, and also on the characteristics of the spin probe. M 12-NSE is an ester of 12-nitroxyl stearic acid and is essentially insoluble in aqueous solution. Liposomes made from dimyristoyl phosphatidyl choline (DMPC) have been used as control lipid bilayer system in which to examine the line shape of this spin probe. Liposomes made from a 1.5% suspension of DMPC in sucrose buffer were serially diluted to give 4 different liposome concentrations. Thus when the liposomes were added to sample tubes each containing 2 nmoles M 12-NSE increased ratios of probe:liposome were obtained. Spectra from these various labelled preparations are shown in Fig. 3. It is quite clear that the water insoluble probe does not remain on the surface of the sample tube but diffuses readily into the liposomes. At the lowest probe:liposome ratio there is no significant contribution to the spectrum from label on the glass surface, although this becomes apparent at very high probe:liposome ratios.

All the spectra have broader lines than those obtained from a methanol solution of the probe, clearly showing that when M 12-NSE is embedded in a homogeneous lipid bilayer, its molecular mobility is restricted. This restraint is believed to be due to the packing of the

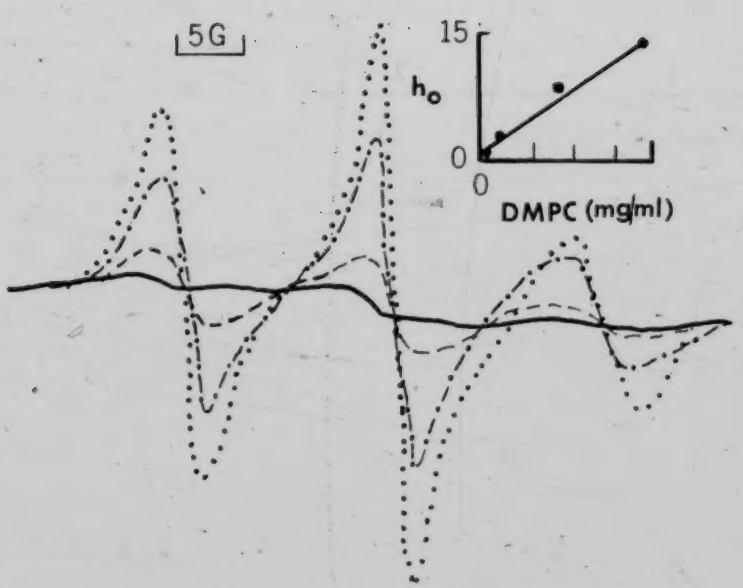


Fig. 3. M 12-NSE in liposomes. The spectra from serial dilutions of DMPC liposomes labelled with M 12-NSE (.....), 5 mg DMPC/ml; (-·---), 2.5 mg/ml; (-----), 0.5 mg/ml; (———), .05 mg/ml. Insert: peak height (h_o) vs. concentration of DMPC in mg/ml.

fatty acid chains of the phospholipid molecules (Smith, 1972; Keith *et al.*, 1973) of the lipid bilayer. As the liposome concentration is decreased there is a corresponding decrease in the intensity of the signal which is seen as a reduction in signal peak heights, but the line shapes remain relatively similar. Since the probe concentration is fixed at 2 nmoles, the different spectra represent increasing probe:liposome ratios. Thus, over the concentration range examined here, the ratio of probe to liposome does not influence the motional freedom of the nitroxide.

Also shown in Fig. 3, is a plot of the peak heights of the midfield lines (h_o) of these spectra and the liposome concentration in mg DMPC/ml. The linear relationship that is apparent again demonstrates that any M 12-NSE present on the glass at the lower liposome concentrations has not made any significant contribution to the spectrum.

4.6 Temperature study of DMPC liposomes

Fig. 4 shows the effects of temperature on spin labelled DMPC liposomes. At 37° the spectrum is essentially isotropic with the three characteristic peaks. The tumbling time calculated with the formula described by Raison *et al.* (1971), which was shown on page reveals τ_o to be less than 5×10^{-9}s. If however the liposomes are cooled the motional freedom of the nitroxyl reporter group is markedly affected. The intensity of all three peaks is reduced; in particular at the lowest temperature shown, 16°C the high field peak cannot be measured with sufficient accuracy. Thus the tumbling time as a measure of the change in bilayer fluidity in this system is not appropriate, and alternate measures were sought. This investigation is dealt with in the following section.

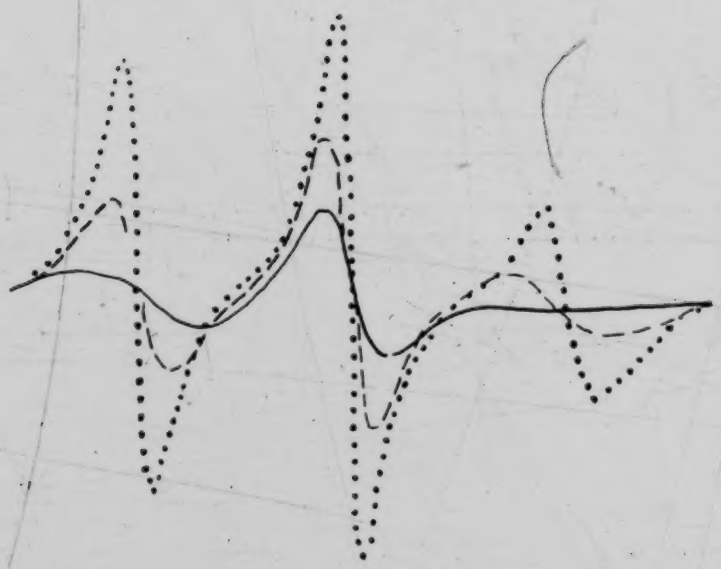
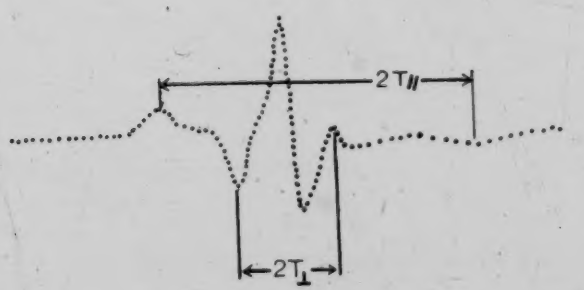


Fig. 4 Effects of temperature on the spectrum of M 12-NSE labelled liposomes. 2.5 mg/ml of DMPC liposomes were labelled as described in text. Spectra were recorded at 37° (• • •), at 20° (– – –) and at 16° (———).

4.7 Quantitative measures

It was mentioned in sub-section 4.1 that isotropic spectra of unrestricted spin labels are frequently quantified by making sue of the formulation of Raison *et al.* (1971). This method does not apply to restricted spectra and in such cases a variety of methods have been derived. Some of these techniques are shown below along with the appropriate references.

a) The order parameter S (McConnell and McFarland, 1970; Seelig, 1970; Hubbell and McConnell, 1971) is most often employed for the quantification of fluidity changes in spin labelled biological membranes. The following illustration is taken from Seelig and Hasselbach, 1971, Eur. J. Biochem. 21:17.



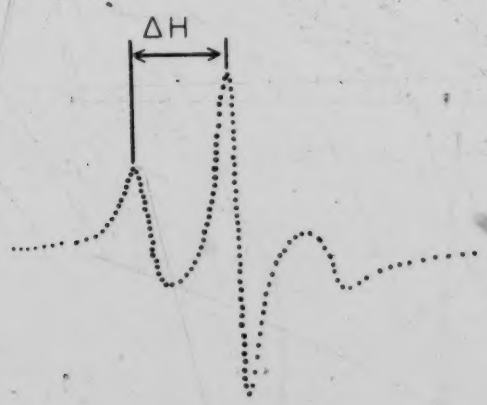
Order parameter, $S = \frac{0.56\ (T_{//} - T_{\perp})}{a}$

$$a = \left(\frac{2T_{//} + T_{\perp}}{3}\right)$$

Similarly Rottem *et al.*, 1973, Biochim. Biophys. Acta 323: 509, have employed $2T_{max}$ and $2T_m$ which if applied to the illustration above, are alternative symbols for $2T_{//}$.

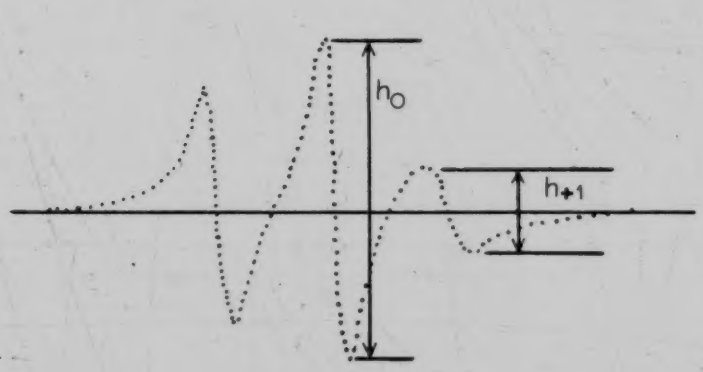
Grisham and Barnett, 1972, Biochim. Biophys. Acta 266:613,

used a parameter they defined as H_{max} which is also an alternative symbol for $2T_{//}$ as shown in the figure above. In a subsequent publication, 1973, Biochim. Biophys. Acta 311:417, these same authors employed another method of quantification suggesting that neither the order parameter S nor the H_{max} could be applied to the experiments being reported. This parameter which was called ΔH is shown below:

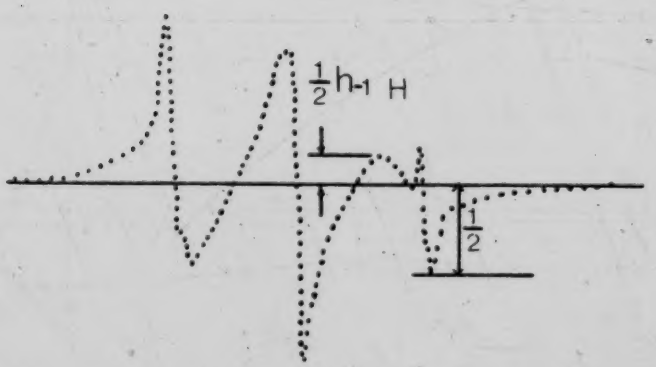


b) The ratios of peak heights have also found use in the quantification of spectra, and some variations are presented below.

A simple ratio of the mid- and the high-field peak heights, as shown below was used by Hegner *et al.*, 1973, Biochim. Biophys. Acta. 307:452, and also by Eletr *et al.*, 1973, J. Mol. Biol. 78:351.

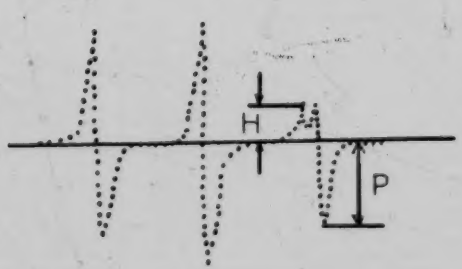


When labelled biological membranes yielded a spectrum with two high field lines, a ratio of the intensity from labels in the hydrophobic region (H) and in the polar region (P) was used. In this approach, half the peak height of the high field line due to labels in the hydrophobic region is denoted by $\frac{1}{2} h_{-1\,H}$ and the corresponding value for labels in the polar region is denoted by $\frac{1}{2} h_{-1\,P}$. This method is illustrated below, and is reproduced from Linden et al., 1973, J. Supramol. Struct. 1:523.



$$\frac{\frac{1}{2}h_{-1\,H}}{\frac{1}{2}h_{-1\,P}}$$

A similar method was employed by Grant and McConnell, 1973, Proc. Nat. Acad. Sci., U.S.A. 70:1238 and by Shimshick and McConnell, 1973, Biochemistry 12:2351. The authors define a "Tempo spectral parameter" which is denoted by 'f' and compares the intensity of peaks from spin labels in the hydrophobic region and in the polar region. This method is illustrated below:



f = "Tempo spectral parameter"

$$f = \frac{H}{H + P}$$

From this list it is apparent that a number of factors can contribute to the type of spectrum, which in turn will determine which method of quantification will be appropriate.. Six methods of quantification were compared using the effects of temperature on the control bilayer, the DMPC liposome. The results are shown in Table 1.

There is a marked effect of temperature on all three spectral peaks with the high field peak (h_{-1}) showing the greatest effect. To compare these different quantitative approaches an inflection point has been derived. This inflection point, (Tc), is the point of intersection of the two straight lines that best fit the non-linear data points obtained, using the computer programme described in section 2.9. The purpose of reporting a calculated value for Tc in Table 1, seeks only to emphasize changes in the slope of plots showing the effects of temperature upon a number of spectral parameters. From the comparison given it can be seen that the different methods of quantification that were used show good correlation. A small difference in the value of Tc obtained with data from the h_{-1} peak is noted. Because of the difficulty of obtaining accurate measurement of this peak at low temperatures, the high-field peak is deemed an unsuitable parameter for spectral quantification of temperature effects when M 12-NSE is employed. Thus in the studies to be reported, evaluation of the spectra has been confined to methods that make use only of the mid-field and low-field peaks, h_0 and h_{+1} respectively. Where the spectrum is typical of a very mobile probe, comparable to a probe solution in methanol, the high-field line has been incorporated into the spectral evaluation.

TABLE 1

The effect of temperature on different spectral parameters of spin labelled DMPC liposomes.

Parameter[a]	40.3°[b]	33.0°	23.1°	19.2°	17.1°	8.6°	T_c[c]
h_{+1}	100	71	42	24	12	7	20.74
h_o	100	83	60	40	26	17	20.91
h_{-1}	100	68	39	18	5	--	19.98
h_o/h_{+1}	100	117	144	169	225	225	20.47
h_o/h_{-1}	100	120	156	226	503	---	18.18
t_o	100	112	184	309	707	---	20.27

a Peak height was measured in mm.

b The data obtained at the different temperature points were expressed as a percentage of the value obtained at 40.3° which was set at 100.

c As determined by computer programme using 10 temperature values from 17° to 40°.

RESULTS

1. Catalytic activity

The interrelationship between temperature and the hydrolytic function of the ($Na^+ + K^+$)-ATPase has been examined by a variety of techniques and the following general conclusions were drawn and listed in the introduction (p. 20):

a) A change in the membrane occurs on cooling.

b) This change usually occurs at about 20°C and is strongly influenced by lipid(s).

c) The temperature induced lipid change alters the function of the enzyme protein, resulting in a higher activation energy for the hydrolysis of substrate ATP.

These general properties have been examined in greater detail for a number of ($Na^+ + K^+$)-ATPase preparations. In the first part of this subsection they will be discussed with reference to membranes enriched in ($Na^+ + K^+$)-ATPase prepared from rabbit renal cortex. Essentially this section will make use of the information contained in the two publications that have been reproduced in Appendix I and II.

1.1 Rabbit kidney ($Na^+ + K^+$)-ATPase

The early work in this laboratory had been carried out with ($Na^+ + K^+$)-ATPase enriched membranes prepared from rabbit renal cortex (Charnock *et al.*, 1971a; Charnock *et al.*, 1971b). This enzyme preparation exhibits the characteristic thermal sensitivity which is frequently demonstrated as a non-linear Arrhenius plot as shown in Fig. 5. The point of intersection occurs at about 20°C and separates two slopes such that the smaller slope or lower activation energy occurs above this point. Extraction of these membranes with the nonionic detergent

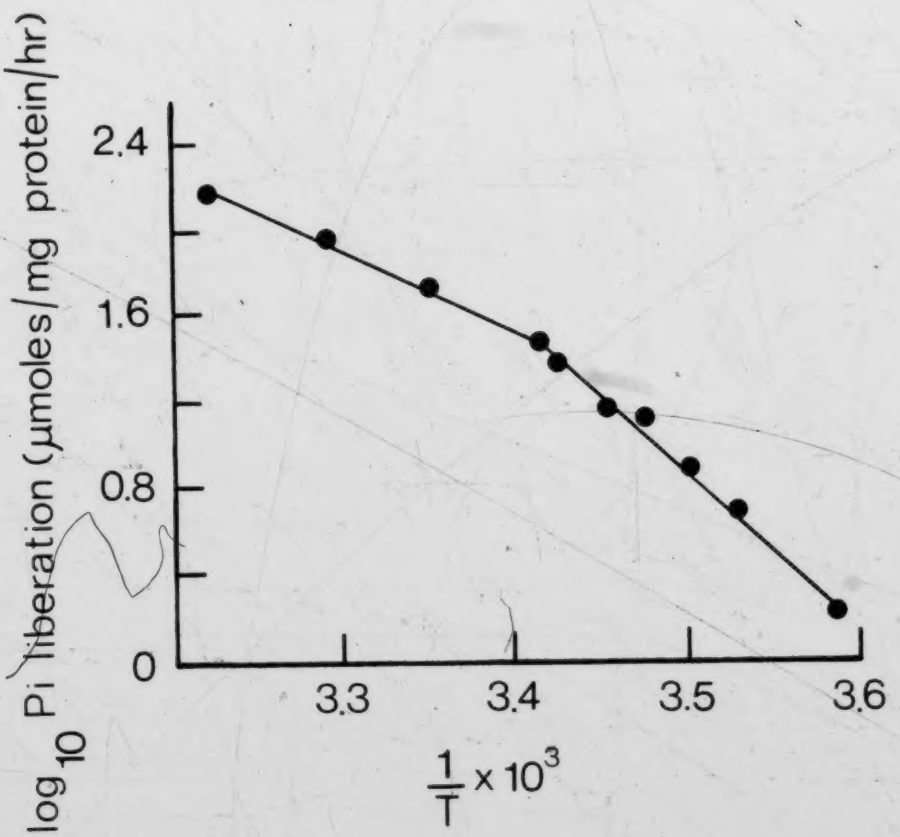


Fig. 5. A typical Arrhenius plot of inorganic phosphate liberation by ouabain-sensitive ($Na^+ + K^+$)-ATPase. The values were obtained as the difference in activity ± 1 mM ouabain with 80 mM Na^+, 20 mM K^+, and 4 mM MgATP. Assays were in duplicate. Activation energies above and below the transition temperature are 14.0 and 30.6 kcal/mole, respectively; from Charnock, Cook and Casey, 1971, Arch. Biochem. Biophys. 147, 323-329.

Nonidet P 40 or with the anionic detergent DOC (0.1%) resulted in a marked increase in the specific activity of the preparation. However the inflection point Tc, was not altered or abolished but remained at about 20°C. Furthermore the values obtained for the apparent activation energies above and below Tc, the Ea_1 and the Ea_2 also did not change appreciably. Therefore the treatment of the enzyme with these two detergents did not result in any detectable change in the temperature dependence of the $(Na^+ + K^+)$-ATPase, although in both cases the specific activity of the enzyme was markedly increased.

A variety of lipases are available for the lysis of membrane lipids. Of these phospholipase-A (PPL-A) removes one of the fatty acyl chains and was employed as a means of altering the membrane lipids of the $(Na^+ + K^+)$-ATPase preparations.

If the membranes were subjected to lipolysis with bee venom PPL-A the thermal response of the enzyme was markedly altered. The effect of temperature on the specific activity of these membranes when presented in the Arrhenius form could be represented by a single straight line with a slope that was distinct from either Ea_1 or from Ea_2. If however these PPL-A treated membranes were reactivated by incubation with phosphatidyl serine (PS), then the characteristic non-linearity was restored and the values for Tc as well as the two activation energies that were now obtained were similar to the corresponding control values.

From these studies it was also determined that the Mg^{++}-ATPase which is frequently associated with the $(Na^+ + K^+)$-ATPase in these membrane preparations was quite different in its sensitivity to temperature, and presumably its interaction with the membrane lipids. In no

case was a linear or non-linear Arrhenius plot obtained, the most appropriate description being curvilinear for such a plot. Thus for the purpose of this dissertation, discussion of the Mg^{++}-ATPase component of the system has been excluded.

With this information the general conclusions stated above, can now be rewritten as they pertain specifically to ($Na^+ + K^+$)-ATPase enriched membranes prepared in this laboratory from rabbit renal cortex:

a) The temperature-activity relationship of the hydrolytic activity of the ($Na^+ + K^+$)-ATPase containing membranes yields a non-linear Arrhenius plot. Thus the hydrolysis of ATP by this enzyme preparation occurs at two distinct energy levels, represented by two different values for Ea_1 and Ea_2. Above a critical temperature Tc, of about 20°C the apparent activation energy (Ea_1) is considerably lower than that (Ea_2) below Tc.

b) The non-linearity can be abolished by pretreatment of the membranes with PPL-A, and subsequent addition of PS restores the non-linearity. Thus it follows that the non-linearity is at least partially a lipid dependent phenomenon, and PS plays some role therein. However, it is not implied that PS is unique in this regard, nor is it specifically located in the membrane with respect to the protein macromolecule.

c) Further enrichment of the ($Na^+ + K^+$)-ATPase by treatment with the nonionic detergent, Nonidet P 40, or with the cationic detergent DOC does not alter either the critical temperature Tc or the two activation energies.

The thermal sensitivity of the ($Na^+ + K^+$)-ATPase was examined further by comparing Arrhenius plots constructed from data obtained by

two different methods, namely activation of the enzyme with cations and the subsequent inhibition of this active state with ouabain. A schematic representation of the methodology is shown below:

A. Enzyme + Mg + ATP = Enzyme + Mg + ATP + ouabain → Basal hydrolysis.

B. Enzyme + Mg + ATP + Na + K → Maximal hydrolysis.

C. Enzyme + Mg + ATP + Na + K + ouabain → Ouabain insensitive hydrolysis.

Therefore, in Method I, Cation Activation = B - A while in Method II, Ouabain Inhibition = B - C.

These two methods of obtaining rates for the hydrolysis of ATP were compared for rabbit kidney membranes treated in six different ways. The results which are summarized in table 2a,b have been discussed in detail in the publications given in Appendix I and II to this dissertation.

For untreated membranes, both methods, cation activation and ouabain inhibition yield non-linear Arrhenius plots with similar inflection points, Tc. The activation energies obtained by these two methods were not however identical; the values obtained for Ea_2 showed a markedly greater difference than the corresponding values for Ea_1. Thus, for the control membranes a comparison of the two methods of determining enzyme activity yields the following observations:

a) Both Arrhenius plots are non-linear.

b) The Tc are similar in both cases.

c) The Ea_1 is slightly higher for the system when assayed by inhibition with ouabain.

TABLE 2a

APPARENT ACTIVATION ENERGY OF $(Na^+ + K^+)$-ATPase AFTER VARIOUS TREATMENTS AS DETERMINED BY OUABAIN INHIBITION OF THE ENZYME

Conditions	No. of Experiments	Ea_1 above $T_c°$[a]	Ea_2 below $T_c°$[a]	$T_c°$
Untreated control	6	19.1 ± 1.3	43.1 ± 3.9	16.5 ± 1.5
Deoxycholate	6	17.8 ± 1.7	36.3 ± 4.2	18.5 ± 2.3
Nonidet P40	13	16.4 ± 0.5	37.2 ± 5.1	19.5 ± 1.8
Phospholipase A	7	23.9 ± 1.5[b]	24.0 ± 3.9[b]	-
Phospholipase A and Phosphatidylserine	5	20.2 ± 1.1	43.6 ± 7.2	18.7 ± 0.5
Phosphatidylserine	3	19.5 ± 1.2	39.7 ± 1.7	20.4 ± 1.2

[a] E_a given as kcal/mole ± standard error.

[b] Values for Ea_1 and Ea_2 are significantly different ($P < 0.05$) from untreated controls.

TABLE 2b

APPARENT ACTIVATION ENERGY OF $(Na^+ + K^+)$-ATPase AFTER VARIOUS TREATMENTS AS DETERMINED BY CATION ACTIVATION OF THE ENZYME

Conditions	No. of Experiments	Ea_1 above T_c°[b]	Ea_2 below T_c°[b]	T_c°
Untreated control	4	15.4 ± 0.7	27.5 ± 1.9	16.7 ± 1.6
Deoxycholate	4	17.5 ± 2.1	30.4 ± 5.9	18.7 ± 1.4
Nonidet P40	4	15.6 ± 0.6	25.6 ± 1.0	19.5 ± 1.2
Phospholipase-A	4	22.0 ± 0.5	36.0 ± 6.7	18.3 ± 2.7
Phospholipase-A and phosphatidylserine	4	21.6 ± 0.5	28.7 ± 1.2	18.7 ± 0.5
Phosphatidylserine	3	17.0 ± 0.7	30.7 ± 1.7	16.2 ± 1.3

[a]Temperature-activity relationship determined by cation activation with 80mM Na^+ and 20 mM K^+.

[b]Values for Ea_1 and Ea_2 given as kcal/mole ± standard error of the mean.

d) The Ea_2 is markedly higher for the system when assayed by inhibition with ouabain.

This pattern was also observed with membranes treated with the nonionic detergent Nonidet P 40 and with the cationic detergent DOC. Once again it is emphasized that the values obtained for Ea_2 reflected the greatest difference between cation activation and ouabain inhibition of $(Na^+ + K^+)$-ATPase. This finding suggested that below Tc, ouabain inhibition of the enzyme is more sensitive to temperature changes than activation of the system by $Na^+ + K^+$.

The most profound difference in the data obtained by cation activation and by ouabain inhibition is observed with membranes treated with PPL-A. As was observed previously, Arrhenius plots of $(Na^+ + K^+)$-ATPase activity determined by ouabain inhibition can now be described by a linear plot with a unique slope. In the case of the cation activation data however, the non-linearity persisted. Moreover, with this latter method, there is also a change in the values obtained for the activation energies but the Tc remains unchanged. If the PPL-A treated membranes are reactivated by incubation with PS, then the data obtained by the two methods of enzyme assay conform to the general pattern described above, namely, a discontinuous Arrhenius plot results. Under these conditions, the values for Tc are similar for the two assay methods, and the Ea_2 for inhibition of the enzyme by ouabain is again markedly greater than Ea_2 for cation activation of $(Na^+ + K^+)$-ATPase.

In summary, the characteristics listed previously for rabbit renal $(Na^+ + K^+)$-ATPase (p. 64) can now be extended as follows:

a) The temperature-activity relationship of membrane bound

$(Na^+ + K^+)$-ATPase from rabbit kidney cortex which is demonstrated by a non-linear Arrhenius plot over the range 5°C to 37°C, can be determined either by cation activation of the enzyme or by inhibition with ouabain.

b) The temperature dependence of the enzyme when determined by ouabain inhibition is greater than when determined by cation activation, particularly below Tc.

c) Liposysis of the membranes by PPL-A reveals that the thermal sensitivity of the enzyme is significantly different when determined by ouabain inhibition or by cation activation. With cation activation the characteristic non-linearity of the enzyme is retained but not with ouabain inhibition. This latter effect of PPL-A can be overcome by incubation of the partially delipidated preparation with PS.

Thus it might be suggested that specific interaction between the protein macromolecule and some phospholipid species is associated with, or is directly responsible for the binding of ouabain (cardiac glycosides), but not the binding of sodium or potassium ions to the membrane-enzyme complex. From this work it cannot be claimed that PS alone is unique in imparting this property.

The conclusions drawn from this work emphasized the urgency of an examination of the effects of temperature on the binding of ouabain to this enzyme.

1.2 Alternate enzyme source

Preliminary experiments on the binding of $[^3H]$-ouabain to the $(Na^+ + K^+)$-ATPase revealed a need for large amounts of enzyme enriched

membranes, and the small yield from rabbit kidney cortex made it imperative that an alternate course of enzyme be obtained. The ox (bos taurus) brain was selected as a possible starting material in an attempt to conserve both the preparation time as well as the cost involved. The following section describes the effects of temperature on the $(Na^+ + K^+)$-ATPase enriched membranes prepared from ox brain, before and after treatment with detergents and with phospholipase-A.

1.3 Ox brain $(Na^+ + K^+)$-ATPase enriched membranes[1]

Although the different methods employed for the preparation of ox brain $(Na^+ + K^+)$-ATPase have been described in detail in the chapter on methods, they are briefly listed below for ease of reading:

Untreated membranes,

four strokes of a teflon-glass homogenizer - method [a]

one 10 s pulse of a Polytron Homogenizer fitted with a PT-20 generator and operated at setting 8 - method [b].

DOC treated membranes,

0.1% DOC for 10 min at 4°C with membranes from [a] and from [b]

0.1% DOC in the presence of 3 mM ATP for 40 min at 30°C with membranes from [b].

0.05% DOC in the presence of 2 mM ATP, 5 mM $MgSO_4$ and 80 mM NaCl for 5 min at 30°C with membranes from [b].

SDS treated membranes,

0.05% SDS in the presence of 4 mM ATP for 30 min at 30°C with membranes from [b].

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[1] Some of the data presented in this section have appeared in press: Charnock, Simonson and Almeida (1977) Biochim. Biophys. Acta. 465, 77-92. See Appendix VI.

The $(Na^+ + K^+)$-ATPase preparations obtained by different treatments were first examined for the effects of temperature on catalytic activity. The hydrolytic activity was determined at 8 or more temperature points between 37°C and 5°C. Since the specific activity of individual preparations varied within any one treatment group, the following standardization measure was employed: the hydrolysis rates obtained at the different temperatures were expressed as precentages of the rate at 37°C and averages determined thereof. These data were used to construct so-called mean Arrhenius plots. From these plots, slopes of the lines were derived to give the Ea_1 and Ea_2 values, and the Tc which was obtained from the point of intersection of these lines. As was described in the methods, a computer programme was used to assist in obtaining these mean values. The results are shown in Table 3.

The results obtained with untreated membranes, whether prepared by method [a] or by method [b] are characteristic of this $(Na^+ + K^+)$-ATPase enzyme. The Arrhenius plot in both preparations is again non-linear with an inflection point, Tc at about 20°C. However the two preparations are not identical with respect to their energies of activation. The less disruptive process, method [a], yields an Ea_1 of 18.1 kcal/mole and an Ea_2 of 37.0 kcal/mole, while the corresponding values for membranes prepared by the more disruptive polytron method are 14.6 and 29.8 kcal/mole. The data do not permit interpretation of these differences at the present time. The differences might signify an alteration in the residual membrane phospholipids, consequent to the methods employed for tissue disintegration. The relationship of the two activation energies is also shown in Table 3, and is represented by a ratio of $Ea_1 : Ea_2$. In both cases this ratio is 0.49.

TABLE 3

APPARENT ACTIVATION ENRGY OF OX BRAIN ($Na^+ + K^+$)-ATPase AFTER VARIOUS TREATMENTS AS DETERMINED BY OUABAIN INHIBITION OF THE ENZYME**

#	Treatment	n	Ea_1 ± SE. kcal/mole	Ea_2 ± SE. kcal/mole	$\frac{Ea_1}{Ea_2}$	Tc ± SE. °C	P < (Ea_1, Ea_2)
1	*Untreated	9	18.1 ± 1.10	37.0 ± 3.40	0.49	20.0 ± 1.00	.001
2	Untreated	4	14.6 ± 1.95	29.8 ± 0.30	0.49	20.9 ± 1.31	.001
3	*DOC, 4°C	6	21.8 ± 0.15	49.2 ± 3.90	0.44	17.8 ± 0.80	.001
4	DOC, 4°C	4	19.7 ± 1.89	41.8 ± 3.08	0.47	17.9 ± 0.93	.001
5	DOC + ATP, 30°C	5	16.4 ± 0.80	45.4 ± 5.40	0.36	16.6 ± 1.83	.001
6	DOC + ATP + Na + Mg, 30°C	3	15.1 ±0.93	32.8 ± 5.30	0.46	19.2 ± 1.53	.05
7	SDS + ATP, 30°C	3	13.6 ± 1.68	30.2 ± 1.20	0.45	20.0 ± 1.27	.01
	MEAN		17.0	38.0	0.45	18.9	
8	DOC, 4°C + PPL-A	3	26.9 ± 1.30	20.3 ± 4.20	1.32	30.8 ± 5.00	.20
9	DOC, 4°C + PPL-A + PS	2	17.6 ± 2.10	35.9 ± 3.45	0.49	22.7 ± 2.40	.05

* Membranes prepared by method (a), all others by method (b).

** Data taken from Charnock et al., Appendix VI.

Data from the five experiments with detergent treated membranes, (four with DOC and one with SDS) all generate Arrhenius plots that are qualitatively similar and are discussed as a single group. The characteristic non-linearity is again common to this group, demonstrating the presence of two distinct activation energies above and below the Tc. In all cases the two slopes reflect significantly different energies of activation above and below Tc ($P < 0.05$). The mean Ea_1 and Ea_2 values for the detergent treated membranes of 17.3 and 39.9 kcal/mole compare favourably with the corresponding values of 16.4 and 33.4 kcal/mole for untreated membranes. The values obtained for Tc fall in a range from 16.6°C to 20.9°C but whether this reflects experimental variability or some specific protein-lipid or other interaction cannot be determined from these data. With one exception, that of PPL-A treatment, the two activation energies of this group display a constant relationship to each other as is apparent from the ratio $Ea_1:Ea_2$. Furthermore, the mean value for $Ea_1:Ea_2$ of this group, (excluding treatment with DOC + ATP at 30°C) is 0.46, showing close similarity to corresponding values obtained with untreated membranes. Treatment with DOC and ATP at 30°C yields a ratio of $Ea_1:Ea_2 = 0.36$, but as was noted above, such differences may be attributed to experimental variability or membrane alteration with equal confidence.

The effects of phospholipase-A on the $(Na^+ + K^+)$-ATPase enriched ox brain membranes are also given in Table 3. The results show that the lipase treated membranes differ from untreated or detergent treated membranes in many respects. The Tc at 30.8°C is considerably higher than is usually reported for untreated membranes enriched with this enzyme, and the values for the activation energies, 26.9 and 20.3

kcal/mole for Ea_1 and Ea_2 respectively are also distinct. The difference in the slopes of these two lines is also reduced ($P = 0.20$) and the ratio of Ea_1:Ea_2 of 1.32 suggests a possible linear rather than non-linear plot for these data. The inverse arrangement of the slopes implies that as a consequence of phospholipase-A treatment the $(Na^+ + K^+)$-ATPase enzyme is now less efficient at the higher temperature. The data presented in this table do not offer an explanation for this unusual observation. The extent of the difference in the behaviour of the delipidated $(Na^+ + K^+)$-ATPase implies a profound alteration of the membrane on exposure to phospholipase-A. If the lipase treated membranes are reactivated by incubation with PS, the variation in the values obtained for Ea_1 and Ea_2, and also for Tc as compared with untreated membranes, is within experimental error. Thus reversal of these effects with this phospholipid suggests that lipase treatment leads to alteration of the lipids rather than a direct effect on the protein moiety of this enzyme.

From the results described in this section a number of characteristics of ox brain $(Na^+ + K^+)$-ATPase can be listed.

a) The enzyme displays a thermal sensitivity that is typical for the $(Na^+ + K^+)$-ATPase.

b) Under the experimental conditions employed, namely 8 to 10 temperature points from 37°C to about 5°C, an Arrhenius plot of hydrolytic activity presents a discontinuous profile which is best described as having two distinct slopes. The slopes intersect at a socalled 'critical temperature' Tc of about 20°C. Above this temperature the enzyme has an activation energy of about 17 kcal/mole and below Tc the requirement is approximately double this value.

c) If the enzyme is purified by extraction with the cationic detergents DOC or SDS, the temperature-activity relationship is qualitatively unaltered.

d) Treatment with PPL-A leaves a unique membrane in which the $(Na^+ + K^+)$-ATPase protein can still function but with a very different temperature-activity relationship from that found in untreated membrane preparations.

e) PS reverses the effects of lipase treatment, thereby implicating the membrane phospholipids in the thermal effects.

From this study it is apparent that the $(Na^+ + K^+)$-ATPase prepared from ox brain membranes is similar to the enzyme obtained from rabbit kidney cortex. It is concluded that in the context of this project, the ox brain is an adequate substitute for the rabbit kidney cortex as a source of $(Na^+ + K^+)$-ATPase.

2. Ouabain binding*

2.1 General

As was alluded to in the introduction and the rationale of this thesis, the binding of $[^3H]$-ouabain to the E-Pi complex was only of marginal interest to this project. Consequently, conditions leading to optimal enzyme phosphorylation, i.e. E_2-P formation were used to study the binding of ouabain to $(Na^+ + K^+)$-ATPase from ox brain. Thus

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*The project to study the binding of ouabain to $(Na^+ + K^+)$-ATPase was done in collaboration, and some of the data presented here have appeared in press: "Variation in sensitivity of the cardiac glycoside receptor characteristics of $(Na^+ + K^+)$-ATPase to lipolysis and temperature", Charnock, Simonson and Almeida (1977) Biochim. Biophys. Acta. 465, 77-92, (see appendix VI). The ouabain binding experiments were carried out by Mr. L.P. Simonson.

all ouabain binding experiments were conducted in the presence of 2 mM Na_2ATP, 2 mM Mg^{++} and 80 mM Na^+. Fig. 6 shows the results obtained when drug binding is measured in the presence of ATP, the activating cations Na and Mg, and 5 x 10^{-7}M [^{3}H]-ouabain. Under these conditions, the rate of binding is very rapid, and remains linear for only a short time before equilibrium levels are attained. When ATP was omitted the equilibrium level of [^{3}H]-ouabain bound to the membranes fell to less than 10%, while in the absence of ATP as well as the cations, there was a further reduction. In the latter situation, the ouabain bound was not significantly different from the background level which was obtained without the addition of enzyme protein. These findings are similar to those of Taniguchi and Iida (1971, 1972).

2.2 Effects of drug concentration and protein concentration on the binding of ouabain.

Using the conditions which are optimal for E_2-P formation, namely 2 mM Na_2ATP, 2 mM Mg^{++} and 80 mM Na^+, binding data were obtained to study the effects of drug concentration on the initial rates of ouabain binding. The results are shown in Fig. 7. From the graph it is apparent that within the range of drug concentrations that were used, 10^{-8} to 10^{-6}M, the maximum rate of ouabain binding to the (Na^+ + K^+)-ATPase receptor was not attained. This is in contrast to the findings of Erdmann and Schoner (1974) who demonstrated that in their experiments with ox brain (Na^+ + K^+)-ATPase preparations the maximum amount of ouabain binding occurred at a drug concentration of 5 x 10^{-7}M. At 1 x 10^{-6}M, the highest concentration that we employed the initial rate of binding was too fast to be measured experimentally, in spite of the fact that the

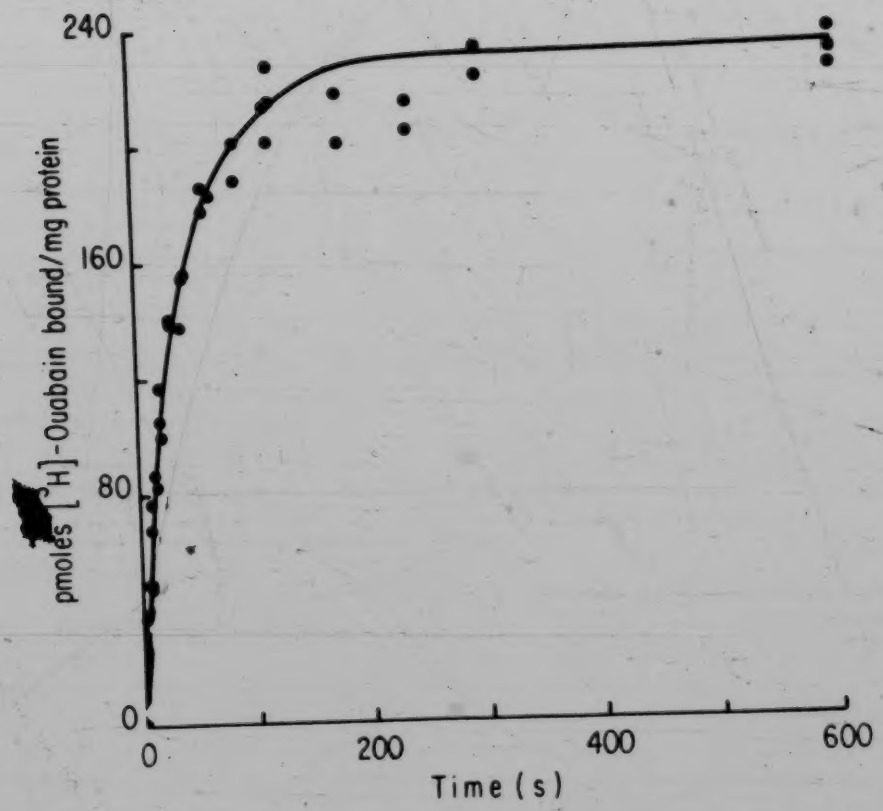

Fig. 6 Progress curve of [^{3}H]-ouabain binding to a membrane preparation of ($Na^+ + K^+$)-ATPase. The enzyme had been treated with 0.1% DOC at 4°C for 10 min. The protein content of this preparation was 128 µg/ml; 78% of the total ATPase activity of the preparation was inhibited by 0.4 mM ouabain and the ouabain sensitive specific activity was 38 µmoles Pi/mg protein/hr at 37°C. The specific activity of [^{3}H]-ouabain was 424 dpm/pmole after dilution with unlabelled carrier ouabain to give a final concentration of 5×10^{-7}M, with 2mM Na_2ATP, 2mM $MgSO_4$ and 80mM NaCl.

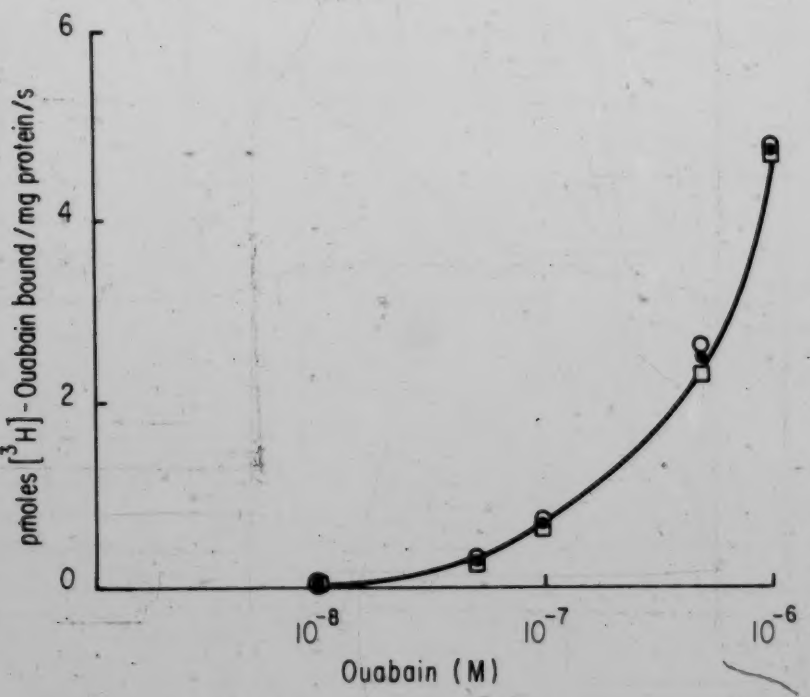

Fig. 7 Rate of [^{3}H]-ouabain binding to a membrane preparation of ($Na^+ + K^+$)-ATPase at 37°C. The enzyme preparation had been extracted with 0.1% DOC at 4°C for 10 min. The specific activity of the preparation was 23 µmoles Pi/mg protein/h; 80% of the total ATPase activity of the preparation was inhibited by 0.4 mM ouabain. Protein concentrations were adjusted to (•), 90 µg/ml; (□), 180 µg/ml and (o), 240 µg/ml. Results are given as pmoles ouabain bound/mg protein/s and are the means of duplicate determinations. Pre- and post- wash solutions contained unlabelled ouabain concentrations equal to the [^{3}H]-ouabain concentration in the incubation medium as described under Methods.

first measurement was taken within 5 s of the addition of the enzyme protein. Therefore for technical reasons the drug concentration necessary for maximum rate of drug-receptor binding could not be determined by this method, despite the fact that five samplings were made within 30 seconds of initiating the reaction. Thus it was important to determine the effect of using possibly a submaximal concentration of 5×10^{-7}M ouabain for our drug binding study. This was done by determining binding rates for different concentrations of protein, and the results are also shown in Fig. 7. Despite a three fold variation in the concentration of the receptor protein, there was no significant difference in the rate of drug binding. That is to say, with respect to the abscissa there is no alteration in the slope of the line and this absence of any shift, would suggest that 5×10^{-7}M ouabain must be close to the maximum concentration required. In this regard it is of interest that a recent paper by Akera and Cheung (1977) describes a method of studying receptor binding reactions which is thought to overcome this problem. Using their methodology on our experiments, Simonson and Charnock (personal communication) have shown that 5×10^{-7}M ouabain yields a maximum value for ouabain binding which is about 5% greater than the extrapolated maximum calculated from the type of probit analysis used by Akera and Cheung (1977).

A second argument favouring 5×10^{-7}M ouabain as an appropriate concentration for drug binding studies can be developed as follows. At lower temperatures the drug binding rate would be slower than at the high temperatures and consequently a drug concentration of 5×10^{-7}M would yield maximal rates. Therefore a complete temperature study from 37°C to about 9°C would make use of maximal initial rates at most tem-

perature points and submaximal rates at the higher temperature points. In contrast, if a higher drug concentration was used, say 5×10^{-6}M, then maximal rates at the lower temperatures would remain unchanged while at the higher temperature points there would arise the possibility that equilibrium binding values would result. It was decided that a temperature study employing a combination of maximal and almost maximal initial rates was preferable to one employing a combination of maximal initial rates and equilibrium binding values. Thus like Erdmann and Schoner (1973a, 1974) drug binding was studied by using 5×10^{-7} M ouabain, which although not maximal at 37°C was technically convenient.

2.3 Drug binding and specific activity[1]

It is generally undisputed that inhibition of the enzyme occurs by the binding of ouabain to the catalytic subunit and the evidence for this belief was documented in the introduction. From this it follows that some relationship should exist between specific activity (μmoles Pi liberated from ATP/mg total protein/hour) of the enzyme and its binding to ouabain. This relationship was investigated using ox brain (Na^+ + K^+)-ATPase enzyme preparations of widely variable specific activity obtained from both untreated and detergent treated preparations. Fig. 8 shows the results of more than 40 experiments, in which binding to these membranes was determined at 37°C. Initial rates were determined during the first 30 s of binding and these are shown in panel A, while equilibrium levels of drug binding which were determined after 10 min are shown in panel B. It is clear that the rate of ouabain binding increases with increasing specific activity of these preparations. The

[1] Some of the data presented in this section have appeared in press: Charnock, Simonson and Almeida (1977) Biochim. Biophys. Acta 465, 77-92, (see appendix VI).

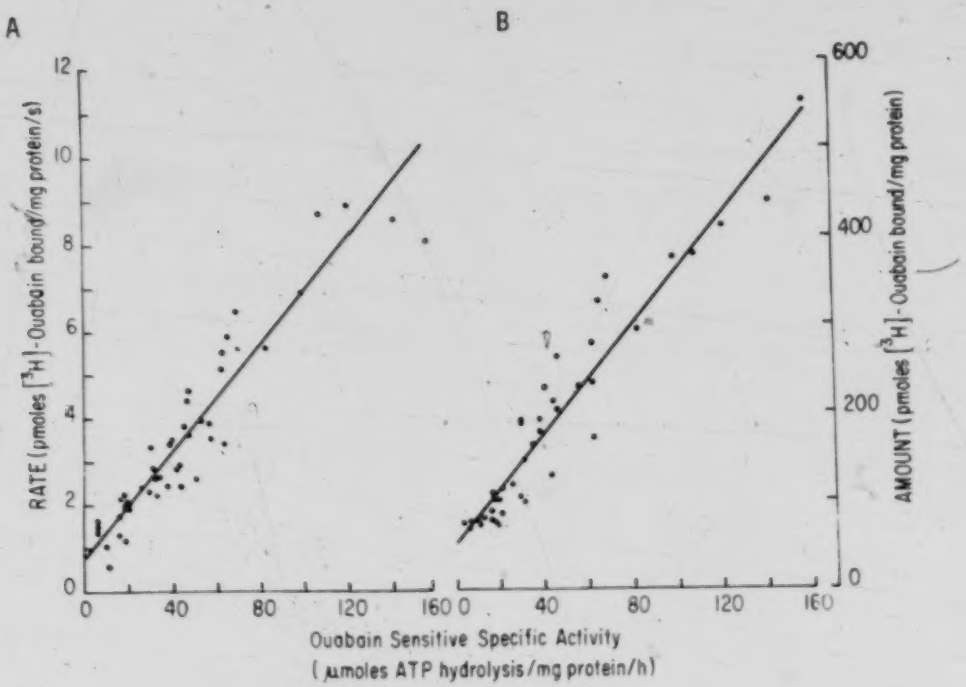

Fig. 8 Relationship of the specific activity of ouabain sensitive ($Na^+ + K^+$)-ATPase with the rate of [^{3}H]-ouabain binding (Panel A) and the amount of [^{3}H]-ouabain bound at equilibrium (Panel B). All experiments were at 37°C. Enzyme preparations of widely different specific activity were obtained from untreated preparations and following the various detergent extractions with DOC and SDS described under Methods. Rates were determined from aliquots taken during the first 30 s after the addition of the enzyme. Each point represents a single enzyme preparation, assays were in duplicate or triplicate. Amounts of [^{3}H]-ouabain binding at equilibrium were determined after 10 min incubation at 37°C and are the means of duplicate assays.

correlation shown is significant at the 0.05 level or better. From panel B it is again evident that a good correlation is also obtained between the amount of ouabain bound at equilibrium at 37°C and the specific activity of the preparation. This latter correlation was also significant at the 0.05 level or better. These results are similar to those of Erdmann and Schoner (1973a, 1974) who suggested that binding studies conducted at equilibrium reflect the number of available ouabain binding sites per unit mass of enzyme, while rate studies reflect the affinity of these sites rather than their number. Thus the data in fig. 8 support the claim made by these authors that both the affinity as well as the number of drug binding sites show a positive correlation with the specific activity.

Despite this positive correlation between the specific activity of the enzyme and the two ouabain binding parameters (equilibrium level and rate of binding), a precise stoichiometric relationship remains to be determined. A major obstacle in its determination is the absence of a precise reference component. Because of this, the rate of ouabain binding has been re-examined with reference to the catalytic sites. Fig.9 (shows a plot of the ratio of two rates namely ouabain binding (μmoles [^{3}H]-ouabain/mg protein/h) and specific activity (μmoles Pi/mg protein/h) versus the enzyme specific activity given as a rate of ATP hydrolysis. From this figure the following observations are made:

a) The line is not parallel to the abscissa therefore the affinity of the receptor for ouabain, changes with the specific activity of the enzyme.

b) The negative slope implies that the affinity of the receptor decreases as the enzyme is purified. Such a change in the

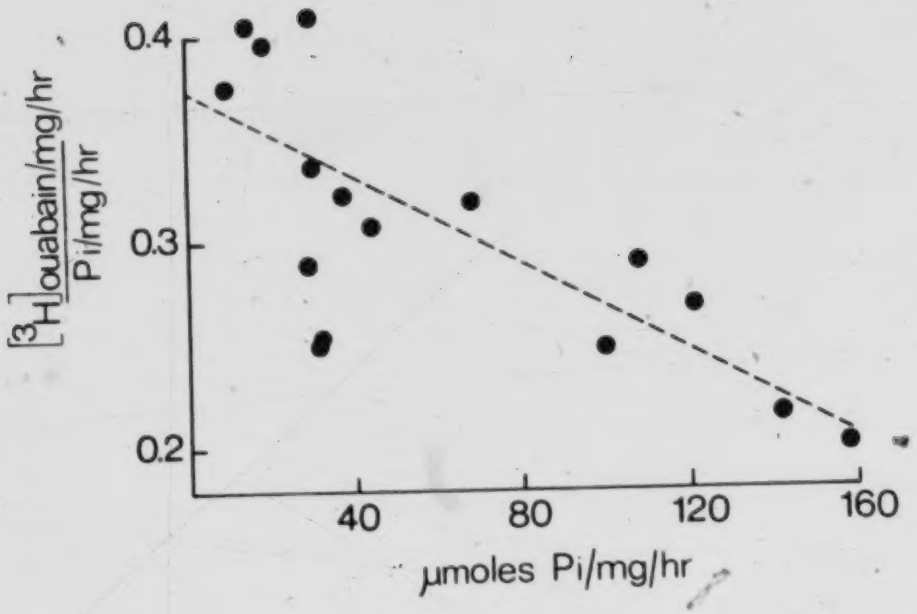

Fig. 9 The effect of specific activity (μmoles Pi/mg protein/hr) on the rate of binding of [^{3}H]-ouabain to ox brain microsomes. The rate of binding of ouabain is expressed as a measure of the specific activity.

receptor may be a consequence of detergent treatment but the data do not permit this degree of interpretation.

c) The absence of a biphasic slope, composed of a horizontal line changing suddenly to a negative slope excludes the possibility that the drug concentration was rate limiting. This reinforces the interpretation that the negative slope of the line reflects a change in the affinity of the receptor.

Therefore from this data, the relationship between the affinity of the receptor for ouabain and the specific activity of the enzyme must at this point remain undefined. This question is discussed again along with other results in a subsequent section of this chapter.

Table 4 shows a comparison of the specific activity and equilibrium levels of bound ouabain bound to the $(Na^+ + K^+)$-ATPase after various treatments with detergent. It is apparent from the table that the specific activity can be gradually increased depending on the extent of the detergent treatment. Mild treatment with DOC at 4° leads to a relatively small increase in the specific activity of the enzyme while a more pronounced exposure to DOC or SDS at 30° in the presence of ATP produces marked increases in the specific activity. When the cations Na^+ and Mg^{++} are present, the purification that results is intermediate to these two extremes. In correspondence with the gradual increase in catalytic sites there is also a gradual increase in the ouabain binding sites following detergent treatment. If however the two parameters are expressed as a ratio then a very different pattern results. It now appears that the drug binding sites decrease rather than increase with the catalytic sites. It is noted however that two of the five treatments yield similar ratios (4.6 and 4.8)

TABLE 4

COMPARISON OF THE SPECIFIC ACTIVITY AND THE EQUILIBRIUM BINDING OF OUABAIN

Treatment	n	Specific[1] activity	Equilibrium[2] binding	EB/SA
None	14	15.2 ± 2.5	90.5 ± 6.1	5.95
DOC 4°	16	31.6 ± 3.2	146.5 ± 12.7	4.64
DOC + ATP + Na + Mg at 30°	7	60.9 ± 4.8	295. ± 26	4.84
DOC + ATP at 30°	3	96.9 ± 18.0	342. ± 55	3.53
SDS + ATP at 30°	3	133.0 ± 17.0	457. ± 51	3.44

1 µmoles Pi/mg protein/hr.*

2. pmoles [^{3}H]-ouabain/mg protein.*

* ± standard error of the mean.

despite the fact that the specific activity of these two preparations showed a two-fold difference. From this data it is not possible to say which of these two treatments is incongruous to the series. Nevertheless the study on drug binding plus the influence of specific activity poses two questions of considerable importance to this project.

a) What molecular mechanism is responsible for the change in the number of ouabain binding sites and possibly their affinity as the enzyme is purified.

b) Does the change in rate of ouabain binding with purification offer a clue to the toxicity of this drug.

3. Ouabain binding and temperature[1]

Earlier work from this laboratory with which I was involved, (see appendix II) had shown that enzyme inhibition by ouabain was influenced to a greater extent by change in the temperature than was the activation of the enzyme by cations. However it was not possible from that work to assess whether the initial rate of ouabain binding or the equilibrium level of drug binding was involved. Thus in pursuing this aspect, the effect of temperature on both these parameters of drug binding was first examined in detail.

3.1 The effects of temperature on the rate of ouabain binding[1].

The effect of temperature upon the rate of ouabain binding to untreated preparations of ox brain enzyme can be seen from the results shown in Fig. 10. As would be expected the rate of drug

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[1] Some of the data presented in this section have appeared in press: Charnock, Simonson and Almeida (1977) Biochim. Biophys. Acta 465, 77-92. (See appendix VI).

Fig. 10 The effect of temperature on the binding of [^{3}H]-ouabain to an untreated membrane preparation of ($Na^+ + K^+$)-ATPase. The enzyme preparation had a specific activity of 16.5 µmoles Pi/mg protein/h; 82% of the total ATPase activity of the preparation was inhibited by 0.4mM ouabain. The protein concentration was 107 µg/ml. The rate of [^{3}H]-ouabain binding was determined from the slope of the line obtained at each experimental temperature, using the Olivetti programme #681009 as described under Methods. The mean initial rates determined in this experiment were from 9.5°C - 36.9°C respectively 0.05, 0.08, 0.13, 0.20, 0.59, 0.89, 1.24 and 1.95 pmoles [^{3}H]-ouabain/mg protein/s. Standard error of the means of each temperature were always <10%. Assays were in duplicate or triplicate.

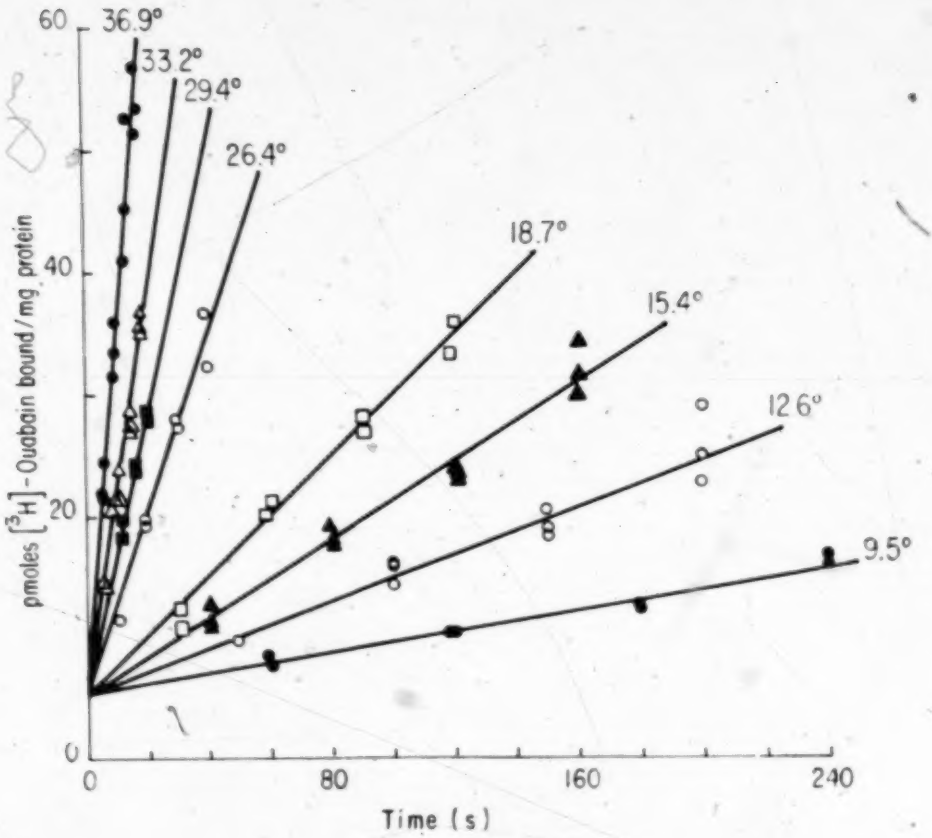

Fig. 10.

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binding decreases as the temperature is lowered. It is also noteworthy that under the conditions of our experiments the rate of binding was linear at all the 8 temperatures that were examined from 36.9° to 9.5°. This supports our earlier observation that a concentration of 5×10^{-7} M ouabain, was suitable for our studies. The mean in the initial rates were 0.05, 0.08, 0.13, 0.20, 0.59, 0.89; 1.24 and 1.95 pmoles [^{3}H]-ouabain /mg protein/s for the different temperatures from 9.5° to 36.9° respectively. The standard errors of the means for the binding rate at the different temperatures were always less than 10%. Experiments such as these were conducted in many subsequent investigations to study the effects of temperature on membranes exposed to different delipidation treatments. In all cases mean rates obtained in this manner were used to construct Arrhenius plots.

From fig. 10, the observation is also made that the linear time period of drug uptake i.e. binding times varied at the different temperatures being less than 30 s at higher temperatures and upto 4 min at 9.5°. The time periods were held to these values in all subsequent experiments.

3.2 The effects of temperature on the equilibrium binding of ouabain.[1]

The effect of temperature on the amount of [^{3}H]-ouabain which was bound to membrane ($Na^+ + K^+$)-ATPase preparations at equilibrium was also examined. The results of a study using untreated enzyme preparations is given in table 5. Since the rate of [^{3}H]-ouabain binding de-

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[1] Some of the data presented in this section have appeared in press: Charnock, Simonson and Almeida (1977) Biochim. Biophys. Acta 465, 77-92, (See appendix VI).

TABLE 5

EQUILIBRIUM LEVELS OF OUABAIN BOUND TO BEEF BRAIN $(Na^+ + K^+)$-ATPase AT VARIOUS TEMPERATURES

Temperature (°C)	$[^3H]$-ouabain bound pmole/mg protein ± S.D.
36.8	87 ± 5.7
33	90.6 ± 2.3
29.3	85 ± 2.5
26.5	94.6 ± 5.2
18.5	94.3 ± 5.2
15.3	89 ± 3.6
12	85 ± 4.8
9.3	93.2 ± 4.2*

In this series of experiments the binding reactions were started by the addition of protein to a final concentration of 225 µg/ml and terminated by filtration after 30 min, except for the lowest temperature* where equilibrium was not reached until 60 min after protein addition. The concentration of ouabain was 5×10^{-7}M in all experiments. The values given are the means ± S.D. of at least 4 experiments at each temperature.

creases with temperature, the binding reaction was allowed to proceed for 30 min in all cases except at the lowest temperature where equilibrium was not reached until 60 min after the reaction had been initiated. The latter was achieved by the addition of membrane protein to a final concentration of 225 μg/ml. The results clearly show that at true (demonstrated) equilibrium, temperature does not affect the amount of drug which can be bound; that is, a variation in the experimental temperature does not alter the number of ouabain receptors available for binding.

3.3 The effects of phospholipase-A on ox brain membranes[1].

The earlier work with rabbit kidney enzyme had shown that phospholipase-A had a marked effect on the specific activity of the enzyme and its sensitivity to temperature. In addition, from the results presented so far it is clear that purification of the enzyme from ox brain, influences the affinity as well as the number of ouabain receptors. In this regard, Taniguchi and Iida (1971, 1973) have reported that treatment of the enzyme with PPL-A resulted in a loss of activity and a reduction in the initial rate of [^{3}H]-ouabain binding. These workers also reported that the binding capacities of the ouabain binding site showed no remarkable change as a consequence of treatment with phospholipase-A. Thus, this lipase plays an important role in the investigation of ($Na^+ + K^+$)-ATPase, and this section discusses these effects in greater detail.

In this examination it was found that the effects of PPL-A varied dependent upon the degree of lipolysis which was obtained and

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[1] Some of the data presented in this section have appeared in press: Charnock, Simonson and Almeida (1977) Biochim.Biophys.Acta 465, 77-92 (See appendix VI).

also on the pretreatment of the enzyme with detergent. These two aspects are discussed separately.

Fig. 11 shows the effects of PPL-A on membranes prepared by Polytron homogenization (method (b), p. 36). In these experiments lipolysis was achieved by exposure of the membrane to 20 units bee venom PPL-A for a period of 40 minutes. Samples were removed at intervals and the membrane enzyme was recovered. Under these conditions, untreated ox brain membranes undergo a biphasic effect. Over the first 5 minutes there is an increase in specific activity of the enzyme, followed by a progressive fall such that within 15 minutes the level has returned to that of the pretreated membranes, and remains unchanged for the remainder of the treatment period. In the case of the ouabain binding there is a corresponding increase in the level at about 5 minutes followed by a fall which remains at a steady state after about 15 minutes. It is notable however, that whereas the specific activity returned to the pretreatment level, the amount of ouabain that was bound at the steady state level was greater than the pretreatment level by about 50%. Thus in contrast to the results of Taniguchi and Iida (1971, 1973) that were cited above, this work clearly shows that lipolysis with PPL-A does not result in a marked reduction in the amount of ouabain that is bound to the $(Na^+ + K^+)$-ATPase.

If the membranes were first extracted with DOC at 4°C, the biphasic effect of PPL-A was lost. This is shown in Fig. 12. After treatment with this detergent the "initial" levels of both ouabain sensitive $(Na^+ + K^+)$-ATPase activity as well as $[^3H]$-ouabain binding are much higher than those of untreated enzyme preparations observed previously. Now the action of PPL-A is to produce an immediate and pro-

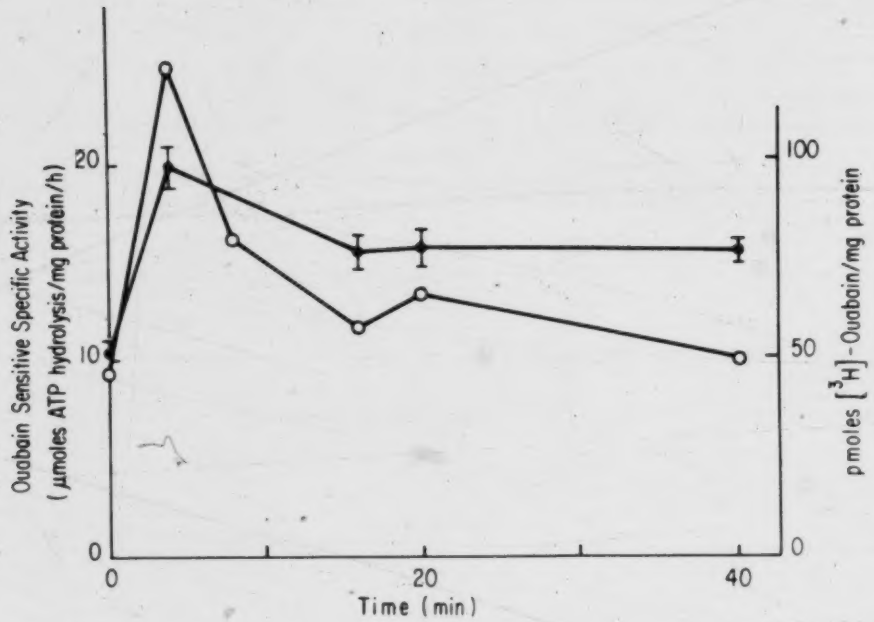

Fig. 11 Effect of progressive treatment at 37°C with 20 units of bee venom phospholipase-A/mg protein on the specific activity and binding of $[^3H]$-ouabain at equilibrium to otherwise untreated membrane preparations of $(Na^+ + K^+)$-ATPase. (•), $[^3H]$-ouabain binding given as pmoles/mg protein, values are the means ± S.E. of triplicate determinations from 1 ml aliquots taken after 5 min incubation at 37°C. (o), specific activity given as µmoles Pi/mg protein/h at 37°C, values are means of duplicate assays.

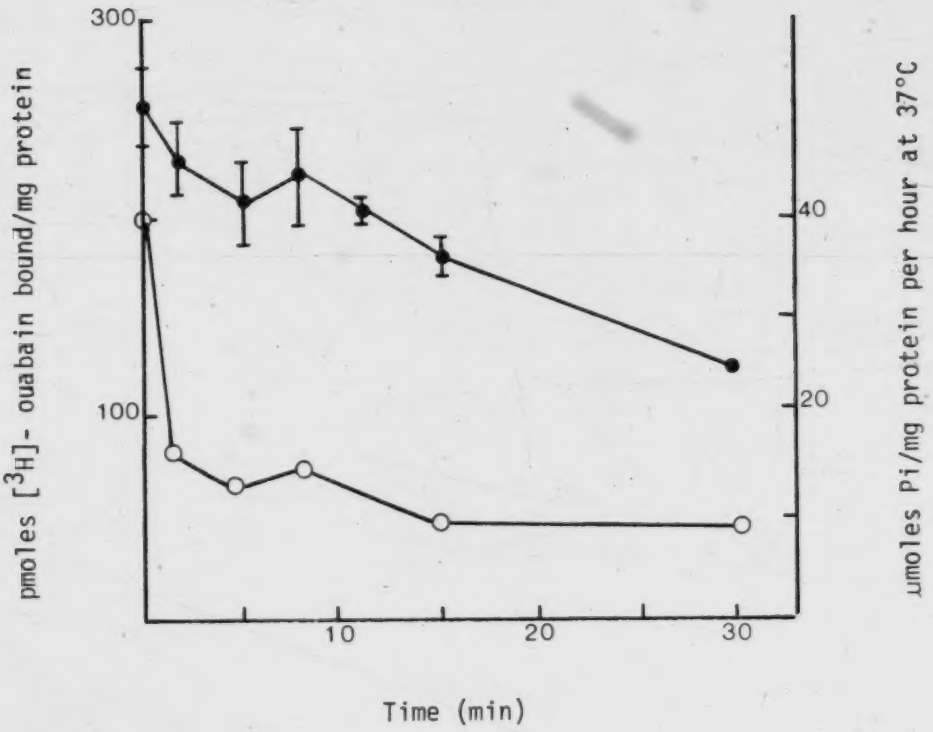

Fig. 12. Effect of progressive treatment at 37°C with 5 units of bee venom phospholipase-A /mg enzyme protein on the specific activity and binding of [^{3}H]-ouabain at equilibrium to membrane preparations of (Na^+ + K^+)-ATPase after 10 minutes extraction with 0.1% deoxycholic acid at 4°C. (●), [^{3}H]-ouabain binding given as pmol/mg protein, values are means ± S.E. of four times 1-ml aliquots taken after 5 minutes incubation at 37°C; (○), specific activity given as µmole Pi/mg protein per h at 37°C; values are means of duplicate assays.

gressive decline in the specific activity of the preparations. It is interesting that the lipase resistant level of the catalytic activity is about the same as that seen with the untreated enzyme preparation, i.e. about 10 μmoles Pi/mg protein/hr. The sensitivity to PPL-A of the ouabain binding sites in these detergent treated membranes is not quite so marked. As the exposure time to lipase is increased, there is a gradual decrease in the amount of ouabain that becomes bound. Thus, as was observed with the untreated membranes, the amount of ouabain bound does not fall in parallel with the specific activity.

A further illustration of this effect is shown in table 6. Here the enzyme which had been prepared by homogenization in a glass mortar with a teflon pestle (method 'a' p. 36) was first extracted with 0.1% DOC at 4°C for 10 min and then subjected to lipolysis with 5 units of phospholipase-A for 5 min. A total of seven preparations were examined for enzyme specific activity as well as the rate and equilibrium level of [^{3}H]-ouabain binding. From the data presented in table 6 it can be seen that the three parameters mentioned above all undergo a change. The activity of the enzyme is most sensitive to lipolysis and is reduced to about 15% of the control value, while the rate and the equilibrium binding of ouabain are reduced to 53% and 73% respectively. That is, both the biochemical activity of the enzyme and the affinity of the binding sites are more susceptible to lipolysis with phospholipase-A than are the number of drug receptors.

3.4 Arrhenius plots of [^{3}H]-ouabain binding to untreated ox brain membranes[1].

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[1]Some of the data presented in this section have appeared in press: Charnock, Simonson and Almeida (1977) Biochim.Biophys.Acta 465, 77-92 (See appendix VI).

TABLE 6

COMPARISON OF THE EFFECTS OF PHOSPHOLIPASE-A ON THE SPECIFIC ACTIVITY OF, AND THE RATE AND EQUILIBRIUM LEVELS OF [^{3}H]-OUABAIN BINDING TO ($Na^+ + K^+$)-ATPase[1]

	n	S.A.[2]	[3]Rate of [^{3}H]-ouabain bound	Equilibrium[4] level of [^{3}H]-ouabain bound
Control	7	26.3 ± 4.3	2.70 ± 0.29	137 ± 23
After phospholipase-A	7	3.8 ± 0.9	1.42 + 0.31	100 ± 18
Difference%		85.4	47.4	27.0

[1]Enzyme was treated with 0.1% DOC at 4°C for 10 min following preparation by method 'a' as described on p. 36.

[2]Mean specific activity in µmoles Pi/mg protein/hr ± S.E.

[3]Mean pmoles [^{3}H]-ouabain bound/mg protein/s ± S.E.

[4]Mean pmoles [^{3}H]-ouabain bound/mg protein ± S.E.

As was described in the chapter on methods two procedures were employed for the homogenization of ox brain, and both membrane preparations were examined for the effects of temperature on the initial rate of ouabain binding. The results, presented as Arrhenius plots are shown in fig. 13. From the mean data obtained from five separate experiments using untreated beef brain membranes prepared by limited teflon-glass homogenization (method a), it was possible to construct an Arrhenius plot for the binding of [^{3}H]-ouabain. This plot is clearly non-linear, and can be described by two straight lines intersecting at a transition temperature (Tc) of about 25°C. Calculation of the apparent energies of activation above (Ea_1) and below (Ea_2) the critical temperature, yields values of 18.5 $\pm$ 2.1 and 29.1 $\pm$ 1.3 kcal/mole respectively. Comparison of these mean values by the paired "t" test indicates significance at the 0.01 level.

The mean data from another group of six experiments using untreated beef brain enzymes prepared by disintegration with the Polytron, (method b) have also been shown as an Arrhenius plot in fig. 13. Once again the plot is non-linear with a similar value for the transition temperature of about 25°C. The mean values for the activation energies Ea_1 and Ea_2, derived from this plot are 20.9 $\pm$ 0.33 and 25.7 $\pm$ 0.86 kcal/mole respectively. It is notable that these values for Ea_1 and Ea_2 are not as widely divergent as was seen with the untreated membranes obtained by glass-teflon homogenization (method 2 above). Comparison of the two slopes by the paired "t" test demonstrated statistical significance at the 0.001 level.

Non-linear Arrhenius plots obtained with data generated by membrane bound enzymes are generally interpreted as phase transitions or

Fig. 13. Arrhenius plots of the rate of [^{3}H]-ouabain binding to ($Na^+ + K^+$)-ATPase from ox brain prepared by method 'a' (▲) and by method 'b' (○). For comparison the rates obtained at 37°C were set at 100% and the experimental values obtained at other temperatures were expressed as a percnetage of this control. To clarify presentation in this form the data from membranes prepared by method 'a' have been offset. The results shown are the means obtained from not less than three experiments.

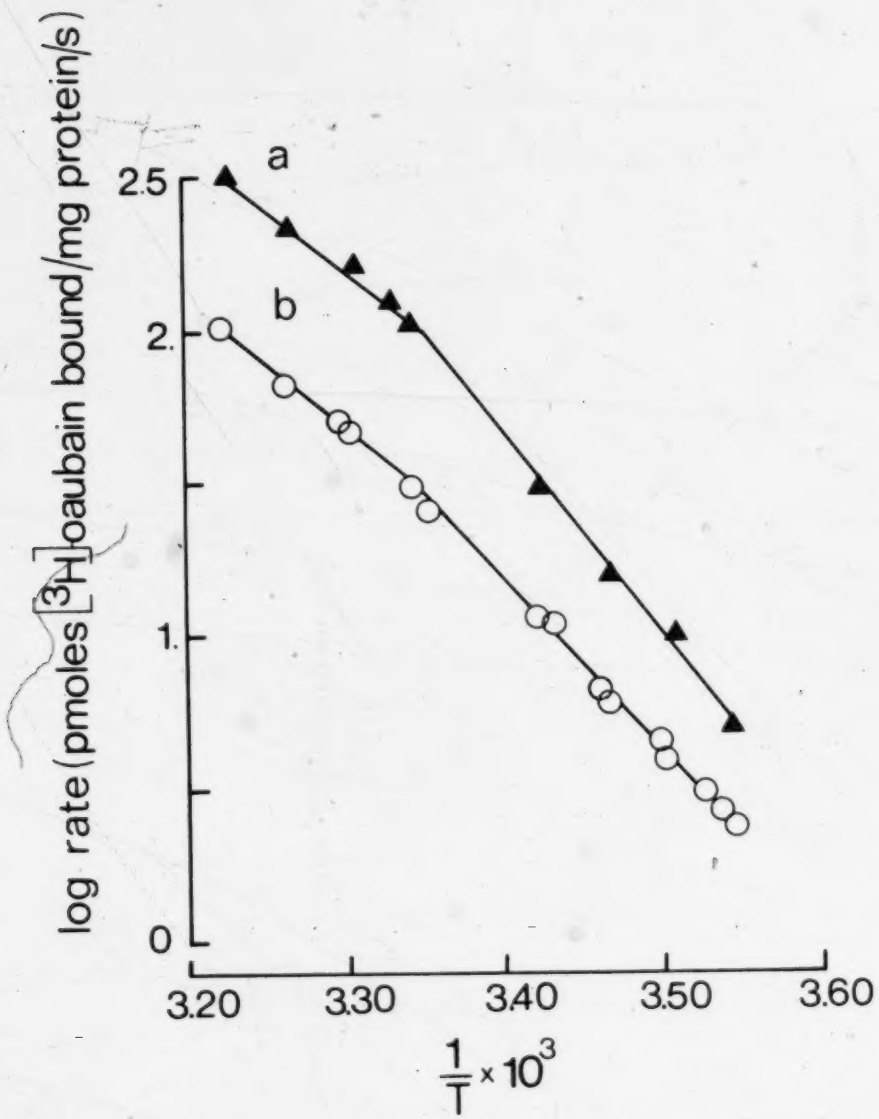

Fig. 13. Legend on separate page.

changes in the lipid bilayer. By analogy it can be inferred from Fig. 13 that the membrane lipids are involved in the binding of [^{3}H]-ouabain to ($Na^+ + K^+$)-ATPase. This supports our earlier suggestion, (Charnock, Almeida and To, 1975) that the cardiac glycoside sensitivity of the ($Na^+ + K^+$)-ATPase is a phenomenon mediated by lipids. It is nevertheless puzzling that the two untreated membrane preparations are not identical with respect to the apparent activation energies. When the latter are expressed as a ratio of $Ea_1 : Ea_2$, the values 0.64 and 0.81 are obtained for the enzymes as prepared by method [a] and method [b] respectively. Since the membranes obtained from method [b] generate an Arrhenius plot that more closely approximates a single straight line, it would appear that with respect to the binding of ouabain, these membranes have lost their temperature sensitivity to some degree. This may be a consequence of the more vigorous disruption of the membranes by the polytron, leading to a preparation with altered residual lipids. If such an alteration is possible, it also implies that the lipids involved in the binding of ouabain to the protein macromolecule can be at least partially removed by this treatment. A second difference in the two enzyme preparations is the increased specific activity of the enzyme prepared by method [b] (see appendix IV). This difference of 5 µmoles Pi/mg protein/hr (15 and 20 respectively for the glass-teflon and polytron preparations) is too small to convincingly implicate the specific activity in the altered temperature sensitivity of the ouabain binding reaction, but not small enough to be ignored.

3.5 Arrhenius plots of [^{3}H]-ouabain binding to detergent treated ox brain membranes[1].

In a previous section (1.3) data were presented which showed that the temperature sensitivity of the catalytic activity of ox brain ($Na^+ + K^+$)-ATPase, varied consequent to the method of preparation. In particular, the enzyme preparation obtained after polytron homogenization, method [b] yielded activation energies, Ea_1 and Ea_2 that were smaller by about 20%; in this respect they bore some similarity to the detergent treated membranes. This lack of correlation between membranes prepared by method [a] and by method [b] was also reflected in the [^{3}H]-ouabain binding studies presented in the section (3.4) immediately above. Thus a similarity exists between the temperature sensitivity of the catalytic activity on the one hand and the binding of ouabain on the other. In order to further examine the effects of temperature on the binding of ouabain, detergent treated membranes were studied.

Fig. 14 shows the Arrhenius plots obtained from a series of experiments which measured the binding of ouabain to membranes that had been treated with DOC under various conditions. If mild detergent extraction is employed, 0.1% DOC at 4°C for 10 min, it reveals differences in the enzyme-enriched membranes obtained by the two different preparative procedures. With enzyme preparations from method [a], there are some obvious quantitative changes following extraction with DOC. The thermal transition in the Arrhenius plot of [^{3}H]-ouabain bound is still clearly apparent, although the mean transition is now somewhat higher

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[1] Some of the data presented in this section have appeared in press: Charnock, Simonson and Almeida (1977) Biochim.Biophys.Acta 465, 77-92, (See appendix VI).

than before, at 29°C ± 1.3°C. There is also an alteration in the values of Ea_1 (11.7 ± 1.3 kcal/mole) and Ea_2 (21.8 ± 2.2 kcal/mole). Nevertheless the non-linearity of ouabain binding to the untreated membrane has remained obvious.

If however enzyme preparations obtained from method [b] were similarly treated and examined the results were quite different. The mean value for Ea_1 at 20.1 ± 1.35 kcal/mole was no longer significantly different ($p>0.4$) from the mean value for Ea_2 = 22.1 ± 1.59 kcal/mole. That is, this form of mild detergent extraction of these beef brain preparations has given a product which no longer clearly displays a marked thermal transition for [^{3}H]-ouabain binding. Preparations obtained by the presumably less disruptive method [a] are more resistant to alteration by this form of detergent treatment than are preparations obtained by method [b].

In addition Fig. 14 also shows the results obtained by further treatment of beef brain enzymes (prepared by method [b]) with DOC at higher temperatures. All these more vigorous procedures also yield data which can best be described by linear Arrhenius plots; the computer assisted Bogartz analysis described in the section on methods does not reveal either statistically significant differences in the activation energies which were calculated for Ea_1 and Ea_2, nor does it yield values for the transition temperatures which fall within the experimental range examined. This linearity of the temperature dependence of the rate of [^{3}H]-ouabain binding can be more easily seen if a complete set of numerical data are presented in tabular form.

Thus table 7 presents numerical data obtained from a variety of ouabain binding studies. In addition to those treatments already

Fig. 14. Arrhenius plots of the rate of [^{3}H]-ouabain binding to (Na^+ + K^+)-ATPase after various treatments. For comparison the rates obtained at 37°C were set at 100% and the experimental values obtained at other temperatures were expressed as a percentage of this control. To clarify presentation in this form the data from the different treatments were arbitrarily offset. Ox brain (Na^+ + K^+)-ATPase prepared by method 'a' after treatment with 0.1% DOC at 4°C for 10 min (▲). Ox brain (Na^+ + K^+)-ATPase prepared by method 'b' after treatment with 0.1% DOC at 4°C for 10 min (■); 0.05% DOC + 2mM ATP + 5mM $MgSO_4$ + 80mM NaCl at 30°C for 5 min (□); 0.1% DOC + 3mM ATP at 30°C for 30 min (○). Results shown are the means obtained from not less than three and sometimes seven experiments.

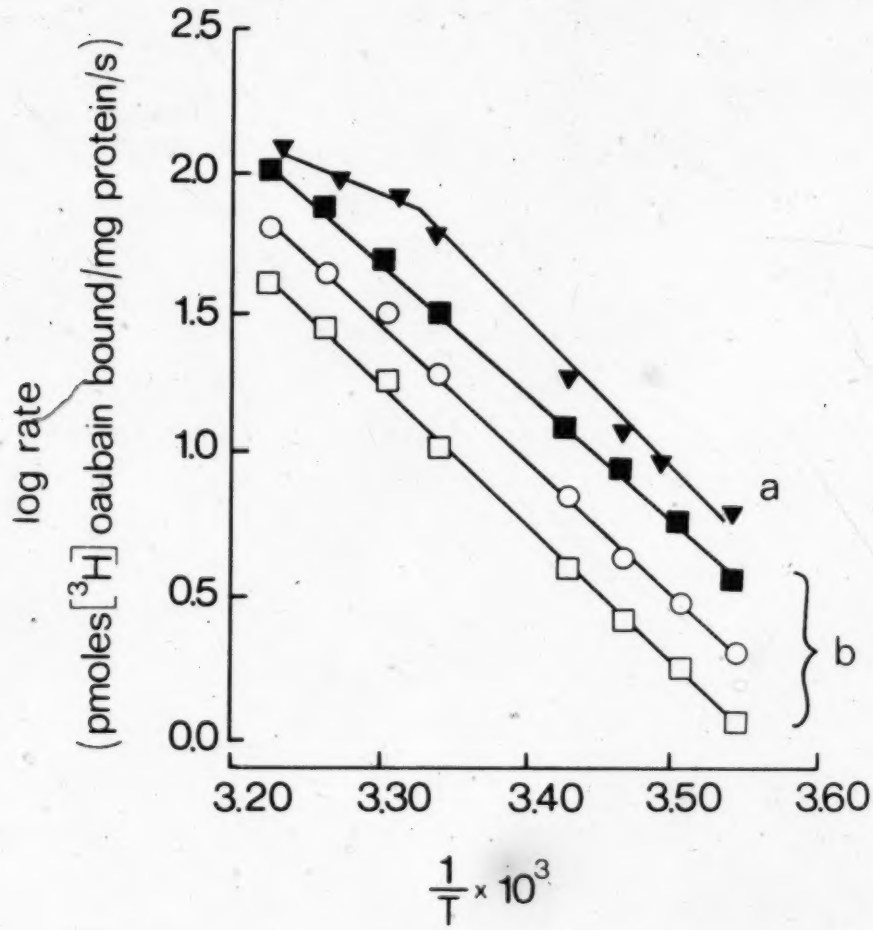

Fig. 14. Legend on separate page.

described in this section data are included from experiments carried out with membranes purified by extraction with SDS. A summary of the observations that can be made from Arrhenius plots of this [^{3}H]-ouabain binding data is listed below:

1) Untreated membranes, irrespective of the method of preparation, yield non-linear plots. Furthermore the values of the Tc are also similar. However, the relationship of the Ea_1 to Ea_2 is noticeably different for the two methods of preparation, Membranes prepared by the more disruptive method (b), yield an Arrhenius plot that approaches linearity.
2) Mild detergent treatment alters the Tc as well as the activation energies of membranes prepared by Glass-teflon homogenization. However the non-linearity is retained.
3) In contrast, mild detergent treatment of the membranes obtained by homogenization with the polytron are no longer significantly non-linear, and the importance and reliability of the value calculated for Tc is decreased.
4) Treatment with DOC at higher temperatures and in the presence of ATP abolishes the non-linearity. If Na^+ and Mg^{++} are present this result is not altered. Furthermore, a similar result is obtained with membranes purified by exposure to SDS at 30°C in the presence of ATP.
5) Treatment of the membranes with phospholipase-A also causes a loss of the characteristic non-linearity of Arrhenius plots of ouabain binding to untreated membranes. Unlike its effect upon specific activity the addition of PS to these partially

TABLE 7

APPARENT ACTIVATION ENERGIES CALCULATED FROM THE RATE OF [^{3}H]-OUABAIN BINDING TO VARIOUS ($Na^+ + K^+$)-ATPase PREPARATIONS AT DIFFERENT TEMPERATURES

#	Treatment	n	$Ea_1 \pm$ SE kcal/mole	$Ea_2 \pm$ SE kcal/mole	$\frac{Ea_1}{Ea_2}$	T ± SE °C	P (Ea_1, Ea_2)
1	*Untreated	4	18.5 ± 2.10	29.1 ± 1.30	0.64	25.4 ± 2.00	<0.01
2	Untreated	6	20.9 ± 0.33	25.7 ± 0.86	0.81	24.6 ± 1.40	<0.001
3	*DOC - 4°C	7	11.7 ± 1.36	21.8 ± 2.20	0.54	29.0 ± 1.30	<0.01
4	DOC - 4°C	3	20.1 ± 1.35	22.1 ± 1.59	0.91	27.2 ± 8.26	>0.40 †
5	DOC + ATP - 30°C	6	20.4 ± 0.56	21.9 ± 0.37	0.93	43.7 ± 3.06	>0.05 †
6	DOC + ATP + Mg + Na - 30°C	5	22.8 ± 0.37	21.5 ± 1.79	1.06	9.5 ± 2.25	>0.50 †
7	SDS + ATP - 30°C	3	21.9 ± 1.84	21.4 ± 1.06	1.02	3.15 ± 3.41	>0.80 †
8	**DOC - 4°C + PPL-A	3	26.5 ± 2.43	25.8 ± 2.31	1.03	-27.0 ± 12.4	>0.80 †
	DOC - 4°C + PPL-A + PS	2	22.2 ± 0.80	24.3 ± 6.25	0.91	40.2 ± 36.7	>0.70 †

* Membranes prepared by glass-teflon homogenization, the remainder with the Polytron PT20.

** 5 units PPL-A for 5 min at 37°C.

†Not significant.

delipidated preparations does not restore the non-linearity.

c) Finally it can be concluded that the binding of ouabain to $(Na^+ + K^+)$-ATPase bears the characteristic thermal sensitivity that is common to its hydrolytic activity. In this case however the temperature effects can be relatively easily altered, as for example by homogenization with the Polytron PT 20, or by treatment with detergents.

4. Spin labelled membranes

4.1 Types of spectra.

Some of the characteristics of the spin probe M 12-NSE were described in the chapter on methods; three properties of this probe are pertinent to this section and are listed below:

a) When examined spectroscopically a solution of this spin probe in methanol yields a typical isotropic spectrum with three sharp peaks of almost equal intensity. Such a spectrum is shown by the broken line in fig. 15.

b) M 12-NSE is practically insoluble in aqueous media. The spectrum of 2nmoles of M 12-NSE, if present as a film on the surface of the glass sample tube, shows no evidence of spin labels in solution.

c) Fatty acid spin labels like M 12-NSE become incorporated into lipid bilayers (Raison *et al.*, 1971; Butler *et al.*, 1974) such that their long axes are oriented perpendicular to the plane of the bilayer (Seelig, 1970; Hubbell and McConnell, 1971).

d) When incorporated into DMPC bilayers, M 12-NSE yields a typi-

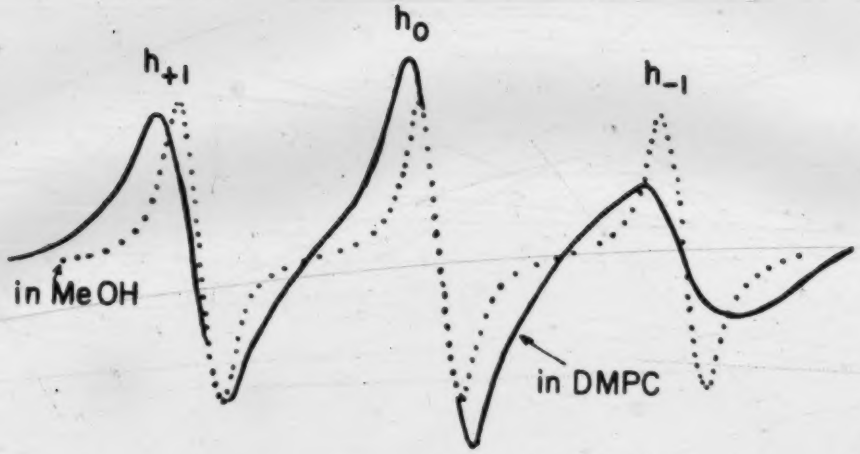

Fig. 15. Types of spectra. M 12-NSE was allowed to diffuse into DMPC liposomes over 10 min at 37°. Samples were introduced into sample tubes which were sealed. Spin labelled liposomes (————) yields a noticeably broader spectrum than does M 12-NSE in solutions in methanol.

cal spectrum that is quite distinct from that of a nitroxide label in solution. In Fig. 15 this is shown by the unbroken line superimposed on the isotropic spectrum of a label in methanol solution (the broken line).

The preliminary ESR experiments yielded a variety of spectra, and these are shown in Fig. 16. From some labelled membrane samples the spectrum was similar to that of an unrestricted nitroxide spin label in solution, while the spectra from other labelled membranes were similar to the restricted spectra of a nitroxide spin label intercalated into a lipid bilayer. However, the majority of spectra from membranes labelled with M 12-NSE were unlike either of these cases, but appeared to be somewhere between these extremes. A number of experimental protocols were therefore undertaken in attempts to resolve this problem, including a variation of the following labelling procedures; temperature at which the labelling was executed, the size of the membrane fragments that were labelled, the storage period of the labelled membrane prior to spectral investigation, the method of enzyme purification and the detergent treatment of the membranes. In spite of this, the type of spectrum that resulted could not be predicted. This problem led to development of the "controlled" labelling technique that was described in the chapter on methodology. With a fixed quanity of spin label present as a film in the sample tube, it was possible to study the effects of different probe:protein ratios with a small amount of M 12-NSE, which at the time these studies were undertaken could not be obtained commercially.

An examination of the results of more than fifty subsequent experiments, revealed that the assorted spectra that were obtained from

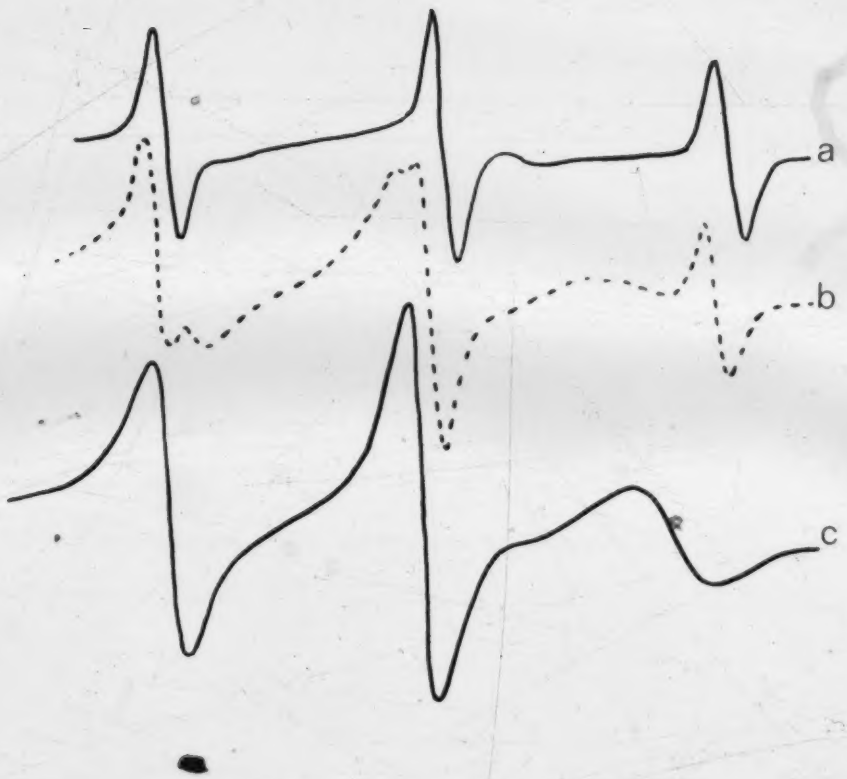

Fig. 16. Types of spectra from ox brain microsomes labelled with M 12-NSE. M 12-NSE was allowed to diffuse into ox brain membranes over 10 min at 37°. The spectra obtained are one of three types, being sharp or unrestricted (top trace, a), mixed (dotted middle trace, b) and broad or restricted (bottom trace, c).

spin labelled ($Na^+ + K^+$)-ATPase containing membranes could be grouped under three headings, which are designated unrestricted, restricted and mixed. Examples of these spectra are shown in Fig. 16a, b, and c. The unrestricted spectrum shown in Fig. 16a is similar to but not identical with, that obtained from M 12-NSE in solution in methanol. Conversely, the restricted spectrum shown in Fig. 16c, is quite different from the unrestricted spectrum, although the three peak symmetry is still apparent. The peaks are now broader and very similar to those obtained with DMPC liposomes, presumably because the nitroxide containing probe experiences some restriction in motion. Fig. 16b shows a mixed spectrum, which as the name implies is a mixture of the two extreme positions, namely the unrestricted and the restricted spectra shown in fig. 16a and fig. 16c respectively. In the mixed spectrum there is a broad absorption, particularly between the low-field and the mid-field peaks. At this stage of the project, the appearance of this secondary peak was random, and its intensity apparently uncontrollable. Attempts to correlate the variation of this peak with any of the parameters that had been examined did not succeed. However, it was recognized that the mixed spectrum reflected both the unrestricted and the restricted spectra. The following section describes experiments carried out in an attempt to resolve this problem.

4.2 Spectral variation and enzyme activity[1].

Using a constant amount of M 12-NSE in each sample tube, a variety of membrane preparations of differing specific activity were

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[1] Some of the data presented in this section have appeared in press: Almeida and Charnock (1977) Biochim.Biophys.Acta 467, 19-28. (See appendix VII).

labelled. At least two membrane concentrations from each preparation were used. Fig. 17 shows a representative sample of some of the different types that were obtained. As was found previously, M 12-NSE will diffuse rapidly from the glass surface into the microsomal membrane. The shortest exposure time that was examined was 2 min at 37°C, after which time no further transfer occurred. In Fig. 17a, a 2-fold difference in membrane concentration (1.25 and 2.5 µg protein/sample tube) is compared. The spectra from both samples have three sharp peaks equally spaced and of similar intensity, not unlike the isotropic spectrum of a rapidly tumbling nitroxide label in a solvent of low viscosity (Jost *et al.*, 1972). This type of spectrum, for which rotational correlation times can be readily determined (McConnell, 1956; McConnell and McFarland, 1970; Raison *et al.*, 1971), is characteristic of relatively unrestricted spin labels in the membrane. The lines in the two spectra have very similar intensities, but the low membrane concentration has some distortion of the base line due to residual M 12-NSE on the surface of the sample tube.

In Fig. 17b, a 4-fold difference in concentration of membrane protein, (4.0 and 16.0 µg protein/sample tube) is compared. Here again the spectra are similar in appearance and resemble the isotropic spectrum described above. There is, however, a difference in the two spectra. A broad absorption is now apparent. This secondary peak is especially noticeable between the low-field and the mid-field peaks. Furthermore it is obtained with the higher membrane concentration, 16 µg protein/sample. However, since the label concentration was the same for both samples, it follows that this phenomenon is only observed at the

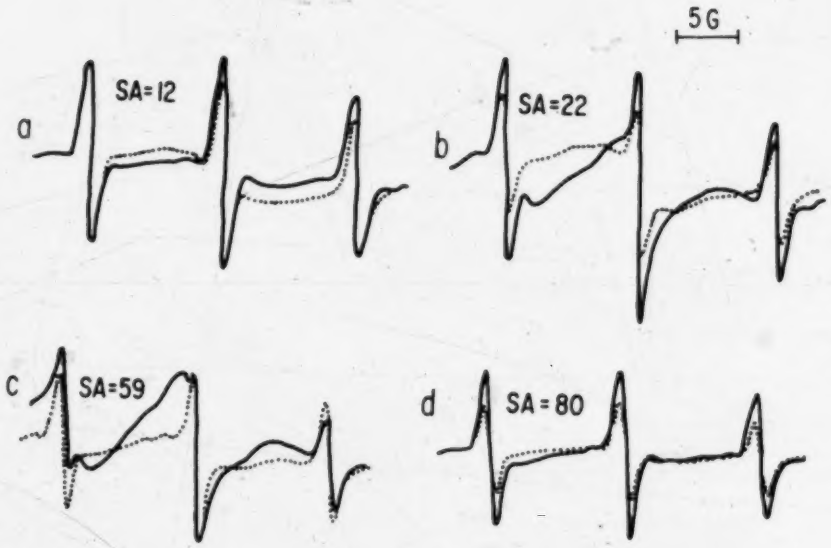

Fig. 17. M 12-NSE in ox brain microsomes. Untreated ox brain microsomes (a,b,c) and after 2 min extraction with 0.1% sodium deoxycholate in the presence of 2mM ATP and 80mM NaCl (d) were labelled in sample tubes containing 2 nmoles M 12-NSE. Diffusion of the probe was allowed to proceed for at least one hour prior to spectral examination. Two membrane concentrations from each of the 4 enzyme preparations were examined. The specific activities (S.A.) of the preparations were respectively 12, 22, 59 and 80 μmoles Pi/mg protein/hr as shown on the figure. The membrane concentrations in μg protein/sample were as follows, and in each set of data the lower concentration is given as the broken line: (a), 1.25 (.....) and 2.5 (———); (b), 4.0 (.....) and 16.0 (———); (c), 6.6 (.....) and 21.0 (———); (d), 2.6 (.....) and 25.5 (———).

lower probe:membrane ratio (1.2×10^{-4} μmoles M 12-NSE:1μg protein). Therefore the increase in membrane concentration from 4.0 to 16.0 μg protein/sample (the only variable) is responsible for the alteration in spectral line shape.

Fig. 17c shows another experiment in which a pair of spectra were obtained with a 3-fold difference in membrane concentration from 6.6 to 21.0 μg protein/sample. At the lower membrane concentration, the spectrum is typically isotropic, but when the membrane concentration in the sample is raised to 21.0 μg protein the spectrum is no longer isotropic. The divergence in spectral line shape is by far the greatest in this pair of spectra although the difference in membrane concentration is intermediate to that used in the two previous experiments, which was 2-fold and 4-fold respectively (fig. 17a and fig. 17b).

Finally, in Fig. 17d a pair of spectra are shown that were obtained by spin-labelling ox brain microsomes that had been extracted with DOC but in the presence of sodium and ATP. The line shape of both spectra are now typically isotropic despite the fact that the membrane concentration of the two samples differs by an order of magnitude from 2.6 μg protein to 25.5 μg protein per sample tube.

From this series of paired experiments it is clear that a number of factors influence the spectrum that will result when ($Na^+ + K^+$)-ATPase membranes are spin labelled. First, the ratio of the number of spin labels and the amount of membrane expressed as the protein concentration, directly influences the spectrum. Second, if the first three pairs of spectra in fig. 17 are examined collectively, it is apparent that in addition to the membrane concentration, the specific activity of the enzyme preparations also influences the spectrum. Finally it

appears that detergent treatment, in this case DOC, can alter both of the above parameters. On the one hand a 10-fold increase in membrane concentration did not alter the shape of the spectrum; on the other hand, although the detergent treatment yielded a more pure membrane, this increased specific activity of the enzyme also did not alter the line shape of the spectrum. That is to say, despite an increased activity as well as an increase in the concentration of the membrane, there is no change in the quality of the spectrum.

4.3 Spectral variation and membrane (protein) concentration[1].

The effects of membrane concentration on spectral type were investigated by examining the spectra of samples containing a fixed amount of probe (2nmole) and varying amounts of membrane from a single enzyme preparation. A selection of these spectra is shown in Fig. 18. Although the spectra again vary with the membrane concentration, they can be grouped into the three catagories of unrestricted, restricted and mixed which was described previously (see section 4.1). At the low membrane concentrations the spectrum is a typical isotropic spectrum characteristic of unrestricted labels. At the very high membrane concentrations, the spectrum is considerably broadened and now resembles the line shape of the spin-labelled DMPC liposomes (shown in fig. 15). At intermediate membrane concentrations, the shape of the spectrum is a superposition of the spectra at the extremes, with the relative intensities being determined by the membrane concentration. Thus at low membrane concentrations, the probe binds to sites that permit greater

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[1] Some of the data presented in this section have appeared in press: Almeida and Charnock (1977) Biochim.Biophys.Acta. 467, 19-28, (See appendix VII).

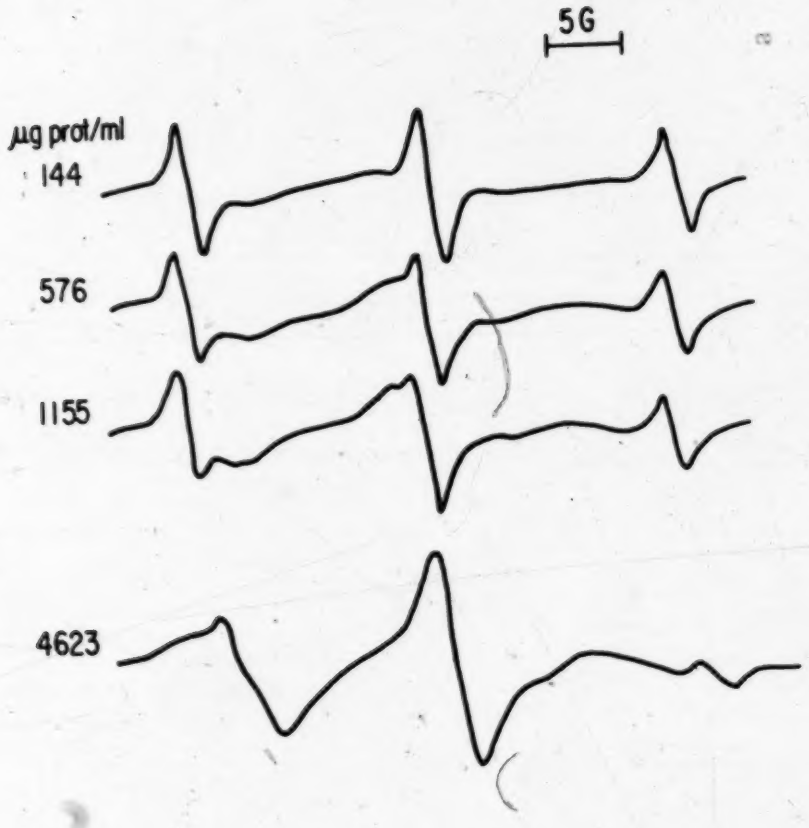

Fig. 18. The effects of membrane concentrations (µg protein) on the spectrum of microsomes labelled with M 12-NSE. Increasing concentrations of untreated ox brain microsomes were added to sample tubes each containing 2 nmoles of M 12-NSE. Diffusion of the probe was allowed to proceed for at least one hour prior to spectral examination. Note the change in spectrum from sharp to broad as the concentration of the membrane increases.

motional freedom, whereas at high membrane concentrations a site that restricts the motion of the nitroxyl is preferred.

From these results and those presented in the preceding section (4.2) an important observation is made: The exact ratio of probe to membrane at which a restricted spectrum is obtained is constant for any one microsomal preparation, but may vary from one preparation to another. It is recalled that results presented in the previous section had shown that the specific activity of the enzyme preparation influenced the line shape of the spectrum. Thus it is possible that this variation probably bears some correlation to the specific activity of the enzyme preparation and also to the method employed in the purification procedure.

Spectra which are intermediate to the two limiting types described above, are a separate group constituting a mixture of two spectral types. They show the three peaks which are characteristic of the three sharp line spectrum and also display evidence of intermediate peaks. At present these mixed spectra cannot be characterized further. Presumably they arise if both restricted and unrestricted labels contribute to the spectrum.

4.4 Effect of enzyme purification on the spectrum[1].

The experiments that have been discussed in the preceding sections strongly suggest that a correlation might exist between enzyme purity and spectral type. Thus it seemed pertinent to examine the

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[1] Some of the data presented in this section have appeared in press: Almeida and Charnock (1977) Biochim.Biophys.Acta 467, 19-28 (See appendix VII).

spectra obtained when a highly purified (most active) membrane preparation was spin labelled. Of the methods that were used for the purification of this enzyme, (and these have been described in the chapter on methods) extraction with SDS was the most effective in increasing the specific activity of the $(Na^+ + K^+)$-ATPase containing membranes. Although the mean values of such preparations was in the range of about 100 µmoles Pi/mg protein/hr, several preparations did attain a level of about 150 µmoles Pi/mg protein/hr. Three such high activity enzyme preparations were spin labelled and examined for their spectral characteristics. Fig. 19 shows the results obtained from a membrane preparation that had a specific activity of 158 µmoles Pi/mg protein/hr. Four different membrane concentrations were labelled with a constant amount of 2 nmoles of M 12-NSE. Although the amount of membrane added was varied from 3.4 to 23 µg protein, it is quite clear that the spectra are all of the restricted type. If the peak height is plotted against membrane concentration, Fig. 20, a linear plot is obtained for the first three points. This relationship supports homogeneous labelling in this system. The highest membrane concentration shows a flattening of the curve, presumably because the amount of M 12-NSE is now the limiting factor. A total of three SDS-treated preparations with elevated specific activities of 158, 144 and 142 µmoles Pi/mg protein/hr, were spin labelled and examined in this manner. In all cases the restricted spectrum was observed with no evidence of mixed or unrestricted spectra.

It is apparent that high activity preparations appear to be homogeneously labelled in that only a single spectral type is obtained. Therefore it is possible that restricted spectra result from the binding of spin labels to distinct sites (R sites). By implication a second

Fig. 19. The effects of membrane concentration after enzyme purification. Increasing concentrations of sodium dodecyl sulfate extracted ox brain microsomes were added to sample tubes each containing 2 nmoles M 12-NSE. Diffusion of the probe was allowed to proceed for at least one hour prior to spectral examination. Spectral comparison of samples containing 22.8 (·····), 11.9 (-·-·-·-), 6.9 (- - - -) and 3.4 (——) µg protein per sample tube.

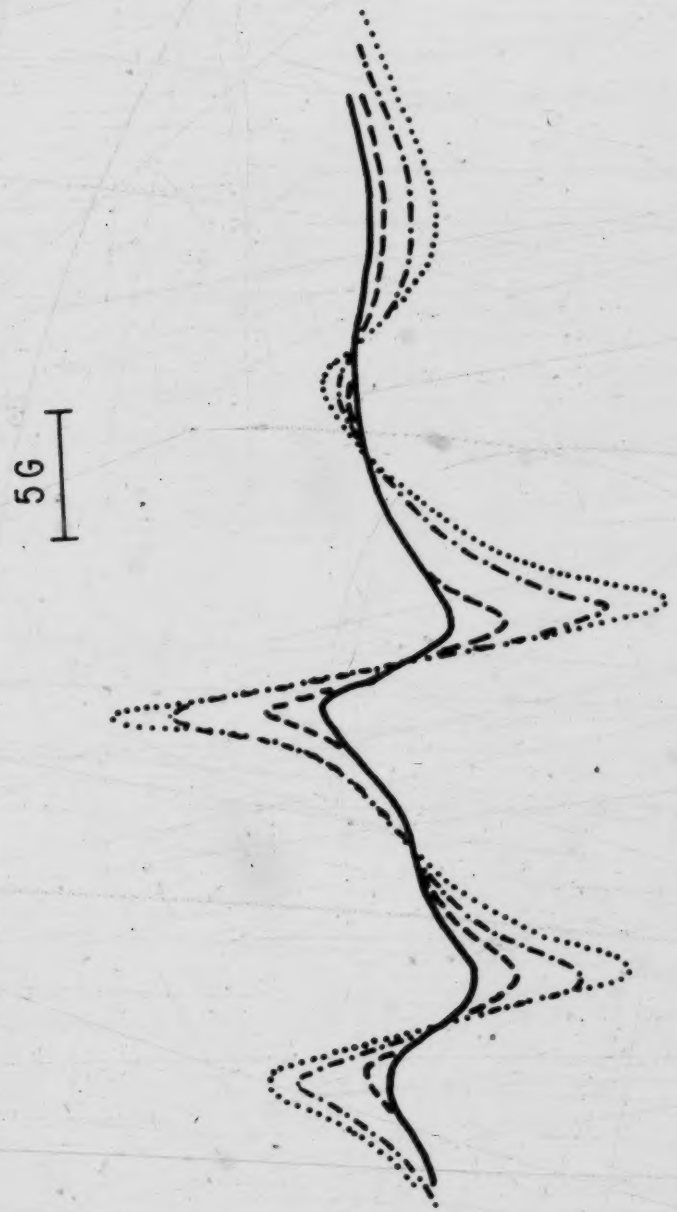

Fig. 19. Legend on separate page.

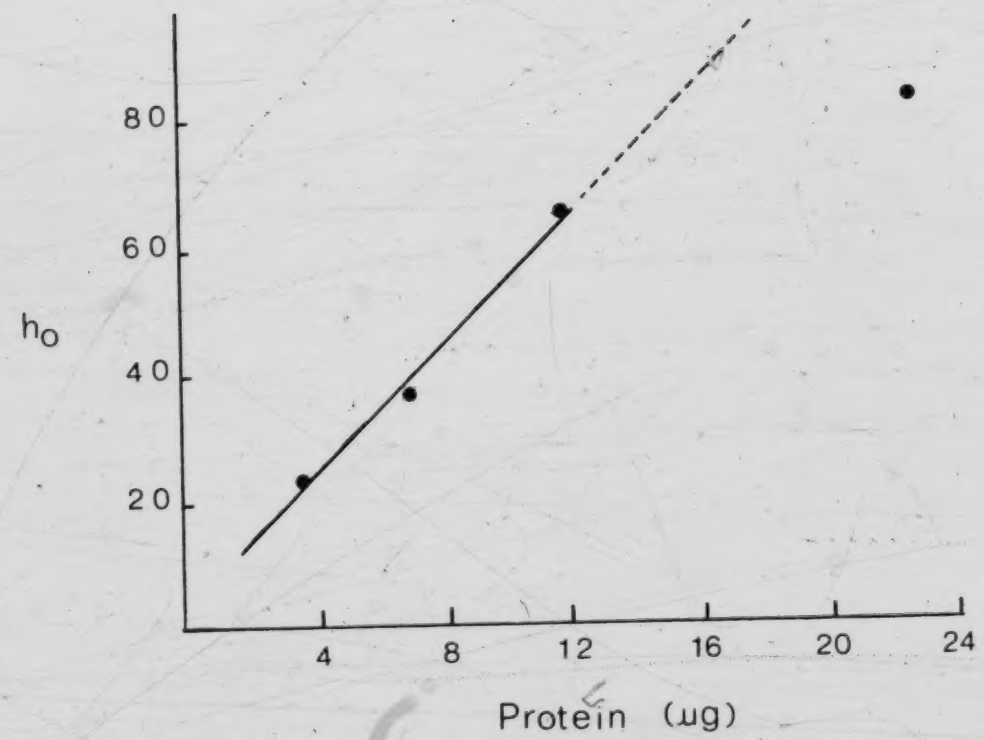

Fig. 20. Spectral homogeneity with membrane (protein) concentration. Increasing concentrations of sodium dodecyl sulfate extracted ox brain microsomes were added to sample tubes each containing 2 nmoles M 12-NSE. Diffusion of the probe was allowed to proceed for at least one hour prior to spectral examination. The mid-field peak height (h_o) is plotted versus protein concentration. There is no change in the profile of the line if the alternate parameter ($h_o \times w_o$) is used in place of h_o.

population of M 12-NSE binding sites would be responsible for generating unrestricted spectra (U sites), while a mixture of R and U site labelling would result in a mixed spectrum. This concept is also supported by the results presented in the preceeding section.

Applying this two site concept to the study described above, two conclusions can be drawn: first, the purification of ox brain membranes by treatment with SDS results in a loss of U sites and a concentration of R sites; second, this treatment results in a parallel increase in specific activity and R sites, inviting the suggestion that the R sites are closely related to the function of the protein macromolecule.

4.5 Spectral variation and temperature

The preceding experiments support the premise that the different types of spectra arise from M 12-NSE binding to two distinct sites. Furthermore, they also define some of the characteristics of the $(Na^+ + K^+)$-ATPase containing membranes, which influence the binding of this probe. Since an important characteristic of this enzyme is its thermal sensitivity, an examination of the effects of temperature on the different spectral categories that have been defined was considered essential. Fig. 21 shows the effects of temperature on the three types of spectra. Microsomes labelled predominantly at the U sites (Fig. 21a) undergo a loss of peak intensity when the temperature is reduced. This line broadening at lower temperature is most pronounced on the high field line. The extent of this change is from 1.3 at 37° to 1.81 at 5° or 138% of the value obtained at the reference temperature, if measured as a ratio of mid- and high-field (h_0/h_{-1}) peaks, or 1.5×10^{-10}s at 37° to 3.6×10^{-10}s at 5° or 240% when measured as tumbling times τ_0.

Fig. 21 The effects of temperature on the different types of spectra. Ox brain microsomes having differing specific activities of $(Na^+ + K^+)$-ATPase (μmoles Pi/mg protein/hour at 37°) were labelled with M 12-NSE as described in the text, and their spectra examined at different temperatures. Preparation (a), specific activity 13 μmoles Pi/mg protein/hr; 1250 ng enzyme protein/nmole M 12-NSE examined at 38° (———) and at 5° (.....). Preparation (b), specific activity 82 μmoles Pi/mg protein/hr; 1515 ng enzyme protein/nmole M 12-NSE at 38° (———) and at 5° (.....). Preparation (c), specific activity 49 μmoles Pi/mg protein/hr; 3250 ng enzyme protein/mole M 12-NSE at 37° (———) and at 7° (.....).

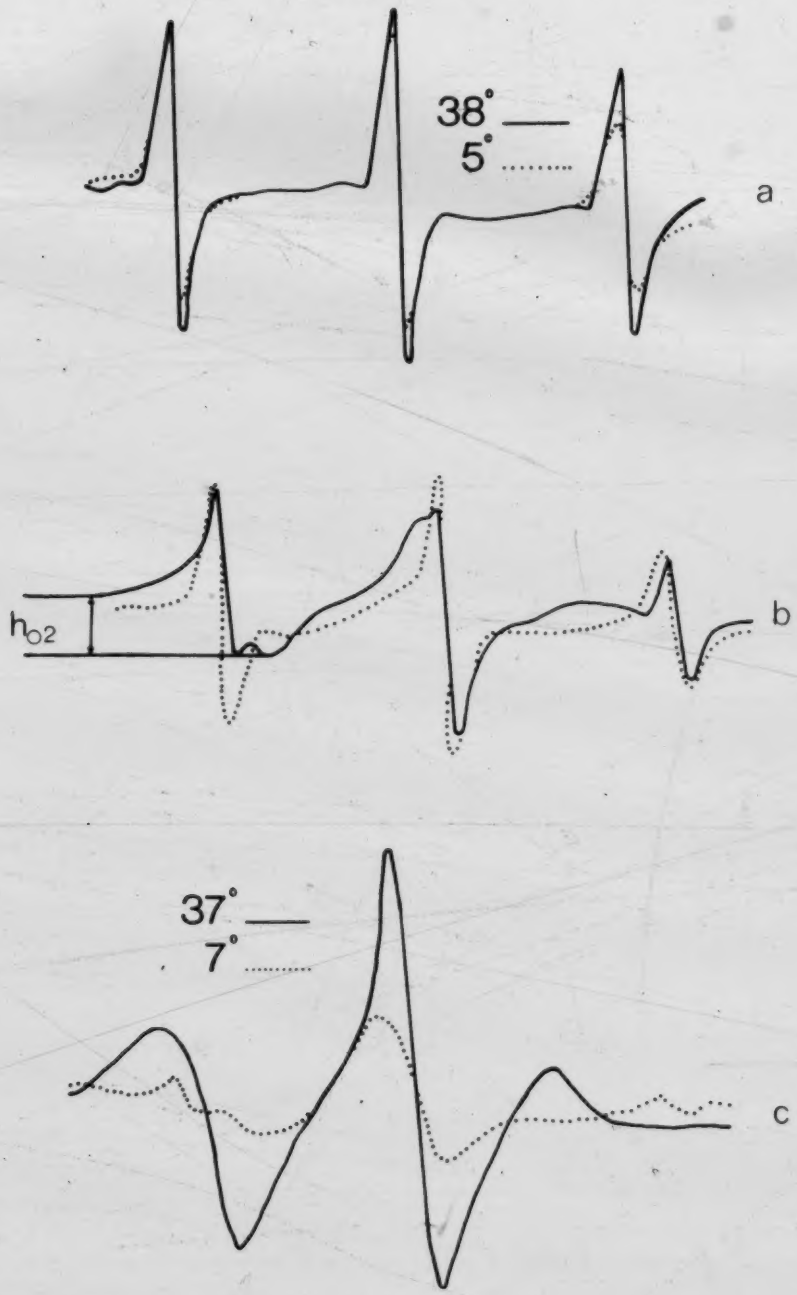

Fig. 21. Legend on separate page.

The corresponding values for M 12-NSE dissolved in methanol are 1.10 to 1.19 for h_0/h_{-1} or 108% and 0.6×10^{-10}s to 1.0×10^{-10}s or 166% for T_o, at 37° and 5° respectively. Thus for a similar change in temperature, M 12-NSE registers a greater change when bound to the U sites on ox-brain microsomes than when free in solution in methanol.

When the R sites are labelled, the changes in the spectrum due to cooling are considerably greater. As was observed with the U sites, Fig. 21C shows that at 7° the high-field peak (h_{-1}) undergoes excessive reduction thereby diminishing the possibility of accurate measurement. Although at the low temperature the spectrum shows some evidence of labelling of U sites, it is important to note that these are relatively few judging from the peak intensities. From these observations it can be concluded that irrespective of the site in the membrane to which it binds, the spin label M 12-NSE experiences some loss of motional freedom when the microsomes are cooled. This effect is considerably more marked at the R sites than at the U sites.

The cooling of microsomes with mixed labelling results in spectral changes that cannot be measured as easily as those described above. On the one hand there is an apparent loss of intensity from labels at the R sites and a concomitant increase in contribution to the spectrum from labels at U sites. Secondly, as was pointed out above, the magnitude of the effect of temperature on the spectra obtained from U and R sites is not equal. Thus changes to the mixed spectrum caused by altering the temperature, cannot be readily applied to an understanding of fluidity changes in the membrane. However it is noted that the secondary peak, h_{02} which reflects probe binding to R sites, de-

creases with temperature. That cooling causes a decrease in the affinity of M 12-NSE binding to R sites is interesting, but in isolation is of little consequence to the present study. However it will be discussed again in a later section.

4.6 Binding affinity of R and U sites.

In the study of the effects of membrane concentration on spectral type, the inference was made that the spin probe M 12-NSE displayed a preference for R sites (section 4.3 and Fig. 18). In this section, experiments which were designed to further examine this possibility, are described. Using a constant amount of M 12-NSE, (2 nmoles/25 μl sample), three different amounts of membrane protein were labelled for each of three preparations with specific activities of 20, 35 and 56 μmoles Pi/mg protein/hr. All spectra were recorded at 37° and were found to be of the mixed type. The results which are presented as a histogram in Fig. 22, show how the height of the secondary peak, h_{o2} (as shown previously by the double-headed arrow in Fig. 21b, varies with the specific activity of the preparation. Two observations can be made from the data given in Fig. 22.. First, as the membrane protein concentration is raised there is a corresponding increase in the intensity of the secondary peak h_{o2}. That is to say, the number of R sites that are available for labelling increases with membrane concentration. Secondly, the labelling of R sites increases disproportionately as the enzyme specific activity of the microsomes is raised. This latter aspect is best seen by comparing the data from ox brain microsomes of lowest and highest activity, at the two extreme protein concentrations shown in Fig. 22. For a similar increase in membrane protein the two preparations register respectively about a 3-fold and a 6-fold

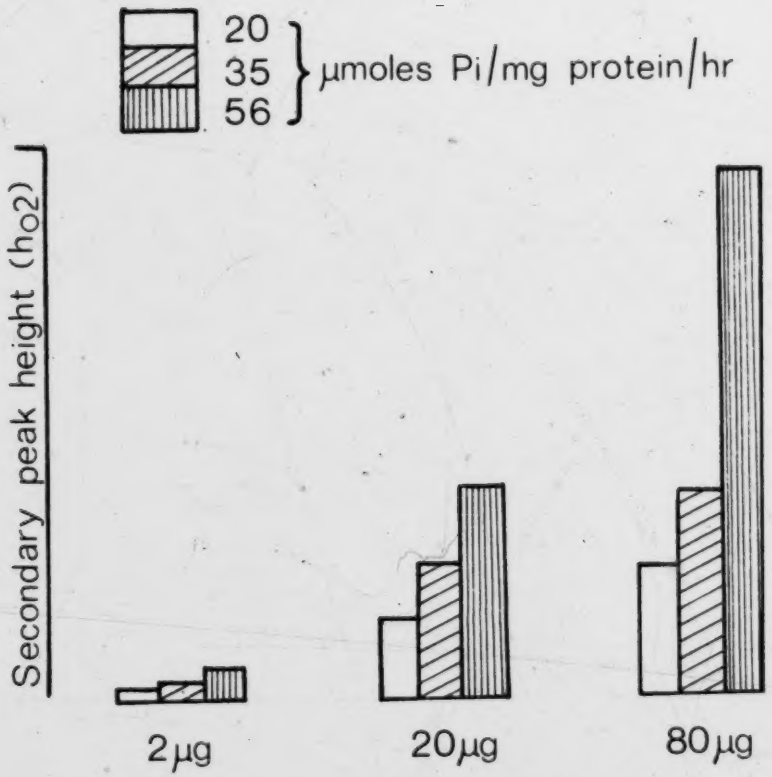

Fig. 22 Probe binding to microsomal R sites. Ox brain microsomes having different levels of specific activity of $(Na^+ + K^+)$-ATPase (shown on the figure) were labelled with 2 nmoles M 12-NSE to produce membrane concentrations of 2, 20 and 80 μg membrane/sample. Both the spin labelling of the membranes and recording of the spectra were conducted at 37°C. The height of the secondary peak h_{02} (as shown in spectra Fig. 21b) was determined for each spectrum and is shown in mm on the vertical axis of the histogram.

increase in the numerical value of h_{o2}. Since the concentration of M 12-NSE is fixed at 2 nmoles, it is reasonable to presume that competition for this label occurs at the higher membrane concentration. Quite clearly, the simultaneous labelling of U and R sites in these experiments prevents precise determination of the affinities for the two sites. Nevertheless, under these conditions it would appear that of the two sites that have been identified, the binding of spin probe to the R sites is preferred. Support for this interpretation can be drawn from the results of experiments previously illustrated in Fig. 21b; these results show that there is an increase in binding to U sites only after a decrease in R site binding occurs due to cooling.

4.7 The effect of ascorbic acid.

The data presented so far support the two-site hypothesis, but do not contribute very much information towards locating these sites. In the case of the R site a reasonable argument can be developed to suggest a location within the bilayer and possibly near the $(Na^+ + K^+)$-ATPase macromolecule. In contrast, information about the location of the U sites is much less definite. It is unlikely that unrestricted spectra arise from labels in solution as this label was shown to be practically insoluble in the aqueous buffer being used. Furthermore, when a spin-labelled microsomal suspension was resuspended in sucrose buffer and then re-sedimented by high speed centrifugation, not only was there no change in the spectral characteristics of the particulate enzyme but the clear supernatant was devoid of any spectroscopic signal. Thus it would appear that the spectra which have been described arise only from spin labels associated with the membrane in some manner. In

this section experiments designed to further characterize this association are described.

Spin labels are rapidly reduced by ascorbic acid and this property has been used to differentiate between spectral peaks produced by labels in solution, from those peaks derived from membrane bound labels (Kornberg and McConnell, 1971; Grant and McConnell, 1973). The results of this investigation are shown in Fig. 23, where it is apparent that M 12-NSE in methanol solution is rapidly reduced by ascorbic acid and this is generally true for nitroxides in solution (Kornberg and McConnell, 1971; Grant and McConnell, 1973). As determined by a measure of the peak height, more than half of the M 12-NSE in methanol solution is reduced within the first 10 min of exposure to 2 mM ascorbic acid. However, rates as rapid as 2 min for 90% reduction of signal have been reported in the literature but these rates are not directly comparable with the result shown here because they can be influenced by a variety of factors including the relative concentration of the nitroxide label to ascorbic acid, and the efficiency of mixing which is attainable in the sample chamber that is being employed.

In the case of the labels at the U sites the reduction in peak height is only half as fast as that seen in the methanol solution, requiring about 20 min for a 50% loss of signal. This difference in sensitivity to ascorbic acid implies that the labels at these U sites may not be freely accessible to this reducing agent. In marked contrast, the labels at the R sites are not significantly reduced by ascorbic acid within the first hour of exposure and only very slightly if the exposure period is monitored for a second hour thereafter. Further-

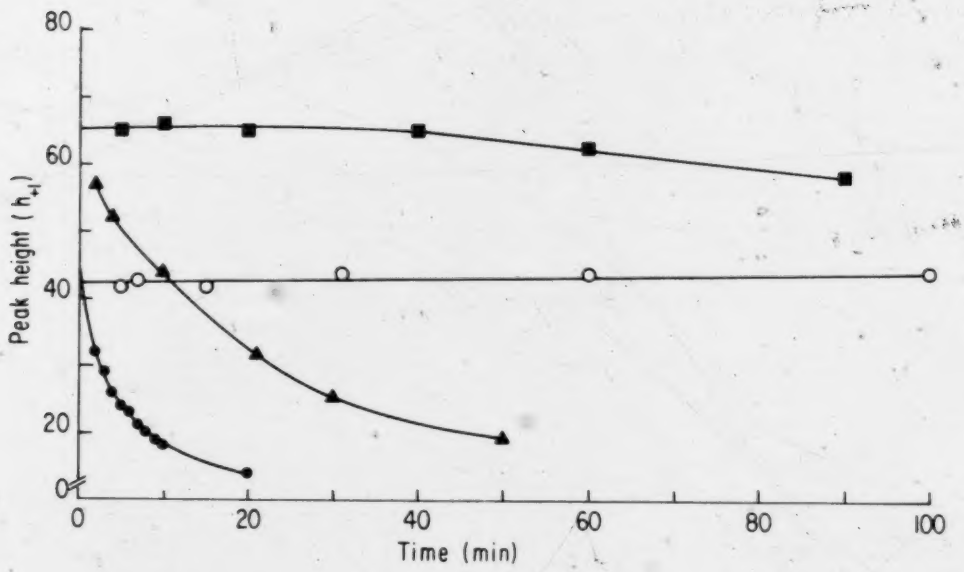

Fig. 23. The effect of ascorbic acid on the peak height of M 12-NSE in different environments. 2mM ascorbic acid buffered to ph 7.5 with Tris was added to M 12-NSE: in methanol (●); at U sites in ox brain microsomes (▲); at R sites in ox brain microsomes (■); and in DMPC liposomes 1 mg lipid/ml(○).

more, when the reduction process was conducted at 0° no potentiation of this slow rate was observed. This protection from ascorbic has been reported by others, and is cited as evidence for a labelled site deep within the membrane core (Kornberg and McConnell, 1971; Grant and McConnell, 1973). A similar very slow rate of reduction was also observed when M 12-NSE labelled liposomes made from DMPC were exposed to ascorbic acid.

4.8 Summary of the evidence for U and R sites.

The results described in the section 4 show that M 12-NSE will bind to $(Na^+ + K^+)$-ATPase enriched ox brain membranes at two distinct sites. If one or other of the sites is predominantly labelled then unrestricted or restricted spectra result. If both sites become labelled, then a mixed spectrum is obtained which is a superposition of the two types seen previously. A summary of the information contained in section 4 can be conveniently presented as a list of the characteristics of the two binding sites and this follows:

a) Labelled U sites are characterized by a sharp spectrum similar to but not identical with rapidly rotating nitroxides in solution, and R sites are characterized by a broad spectrum similar to nitroxides within a lipid bilayer.

b) Both sites are influenced by the ratio of the probe to membrane concentration; a high ratio results in the labelling of U sites while a low ratio results in R site labelling.

c) Enzyme preparations with high specific activity yield spectra resulting from R site labelling while those with low specific activity yield spectra typical of U site labelling. It

is possible that very pure ($Na^+ + K^+$)-ATPase preparations lack U sites altogether but such preparations have not yet been obtained in this laboratory. However, mild treatment of ox brain membranes with DOC may cause an increase in enzyme specific activity without a loss of U sites.

d) Both U and R sites report a loss of mobility when labelled ox brain membranes are cooled. The effect of temperature is considerably greater on the R sites.

e) M 12-NSE has a higher binding affinity for the R sites than for U sites. That is, when a limited amount of spin label is available, R sites are preferentially labelled. In support of this observation, mixed spectra when cooled show a decrease in R site labelling and an increase in the labelling of U sites.

f) Ascorbic acid, reduces labels at U sites fairly rapidly, but not as fast as labels in solution in methanol. M 12-NSE at R sites is relatively resistant to reduction by 2mM ascorbic acid.

Although the precise location of the U and R sites with respect to membrane topography is not known, their marked diversity as documented above, warrants a detailed study of the effects of temperature. These studies are presented in the following section.

5. The effects of temperature on spin labelled membranes

In the chapter on methods, section 4.7 contained a discussion of some of the quantitative approaches that have been used in biological applications of ESR. Some of the important points are pertinent to

this section and are reproduced below:

a) A number of factors can contribute to the type of spectrum, and these in turn may influence the choice of method used for quantification of spectral changes.

b) Six methods of quantification were compared using DMPC liposomes labelled with M 12-NSE, as a control lipid bilayer.

c) From the investigation it was decided that when the spectrum is broad, like that of a probe in a synthetic bilayer, a ratio of the mid- and low-field peaks [h_0/h_{+1}] is an appropriate quantitative measure.

d) When the spectrum is typical of a very mobile spin probe, comparable to a probe solution in methanol, the ratio of the mid- and high-field peaks [h_0/h_{-1}] is an appropriate quantitative procedure.

e) The purpose of reporting a calculated value for an inflection point (Tc) is only to emphasize changes in the slope of plots showing the effects of temperature on the spectrum.

5.1 The effects of temperature on U sites.

When U sites on ox brain microsomes are labelled with M 12-NSE the spectrum obtained is similar to that seen with these nitroxyl spin labels in solution, and the suggestion was made that one possible location might be the surface of the bilayer (Grant and McConnell, 1973). The uniqueness of this site may result from specific phospholipid head groups in this region which in turn could influence the transition temperature (Almeida and Charnock, 1977). Thus it was of peripheral interest to examine the effects of temperature on labelled U sites. Fig.

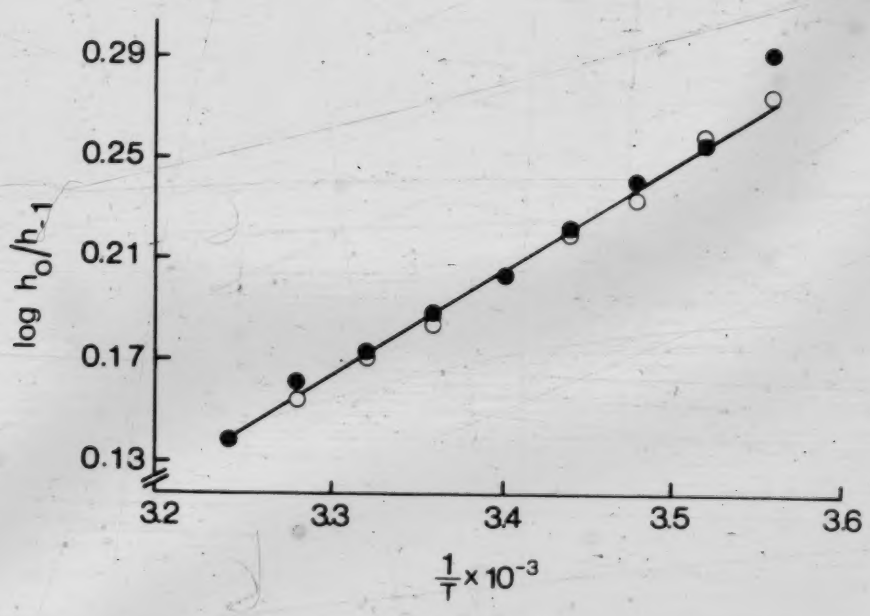

Fig. 24. Arrhenius plot of spectral changes from spin labelled unrestricted (U) sites on ox brain membranes labelled with M 12-NSE. The spin probe was allowed to diffuse into the membranes which were then equilibrated overnight at 4°C. About 30 µl of the labelled membranes were introduced into capillary tubes (1.1 mm internal diameter) which were then sealed. Temperature alteration was achieved by cooling the sample in multiple steps of a few degrees between 37° and 5°C. The plots represent mean values of 9 determinations for each condition; (●) untreated membranes, and (○) sodium deoxycholate extracted membranes. A linear thermal response was found under both experimental conditions.

24 shows Arrhenius plots of the peak height ratio h_o/h_{-1} obtained from labelled ox brain microsomes, both before and after extraction with DOC. It is quite clear that M 12-NSE at U sites, although it reports a change in fluidity with temperature, provides no evidence of any discontinuity over the range examined. Furthermore, this linear response to temperature is not changed by extraction of the membranes with DOC although this treatment is known to remove membrane lipids and to result in a marked increase in the specific activity of the enzyme. However, it should be recalled that the temperature dependence of enzyme activity is not altered by this detergent treatment.

Thus while U sites definitely report a sensitivity to temperature, it does not appear that the label in this position reports any thermal transitions which could be attributed to changes in the physical properties of membrane lipids.

5.2 Limited study of the effects of temperature on R sites.

The results presented in section 4.2, 4.4, and 4.6 had shown a positive correlation between enzyme specific activity and the amount of R sites. Thus it is possible that selective retention of some specific lipid or lipid array might result from the purification procedures that were employed. Thus it would follow that spin labels at R sites, might report lipid sensitivities to temperature from a site in the membrane that is near or related to the enzyme protein macromolecule. On the strength of this supposition, the effects of temperature were examined on labelled R sites, using ox brain membranes both before and after extraction with detergents.

As shown in Fig. 25, when R sites are labelled, the spectrum is of the so-called 'broad' type similar to that obtained with lipo-

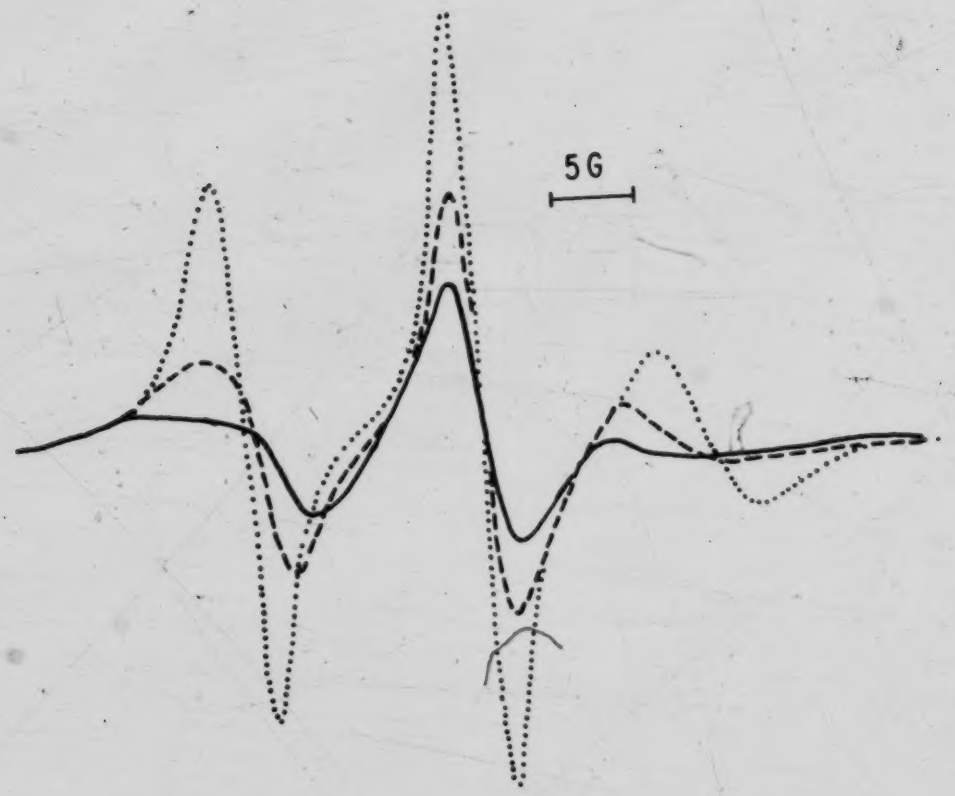

Fig. 25. The effects of temperature on the spectrum of M 12-NSE bound to restricted sites on ox brain membranes. M 12-NSE was allowed to diffuse into ox brain membranes which were then equilibrated overnight at 4°C. About 30 µl of the labelled membranes were introduced into capillary tubes (1.1 mm internal diameter) which were then sealed. Temperature alteration was achieved by cooling the sample in multiple steps of a few degrees between 37°C and 5°C. Three representative traces obtained at 37° (· · · ·), at 19° (- - -), and at 7°C (———) are shown.

somes made from the synthetic phospholipid DMPC as was shown in Fig. 4 (p. 57). Furthermore, lowering the temperature results in a similar decrease in intensity of the three spectral peaks. Again, as was shown previously in the study with DMPC liposomes, the high-field peak experiences the greatest effect of temperature. Qualitatively it appears that labelled R sites in ox brain microsomes are similar to labelled DMPC liposomes. Fig. 26 gives the Arrhenius plots that were obtained with data from untreated and detergent treated enzyme preparations. Unlike the plots obtained for labelled U sites, it is very clear that none of the present plots is linear. The computer program described in section 2.8, p. 41 was used to obtain inflection points, all of which occur between 21-22°C, with no distinction with respect to the different treatments. The values for these inflection points and for the respective slopes of the plots above and below this point are very similar for the different treatments. This finding exactly parallels the effects of these detergents upon the thermal dependence of $(Na^+ + K^+)$-ATPase activity, but does not provide unequivocal evidence in support of the concept of increasing lipid specificity with increasing purification of the enzyme.

5.3 Extended study of the effects of temperature on R sites in untreated membranes.

The studies described above, rely upon a limited number of data points within the temperature range examined. Thus it is possible that secondary and perhaps less marked inflections might be obscured. These studies of the effects of temperature on M 12-NSE labelled ox brain microsomes have therefore been repeated in much greater detail.

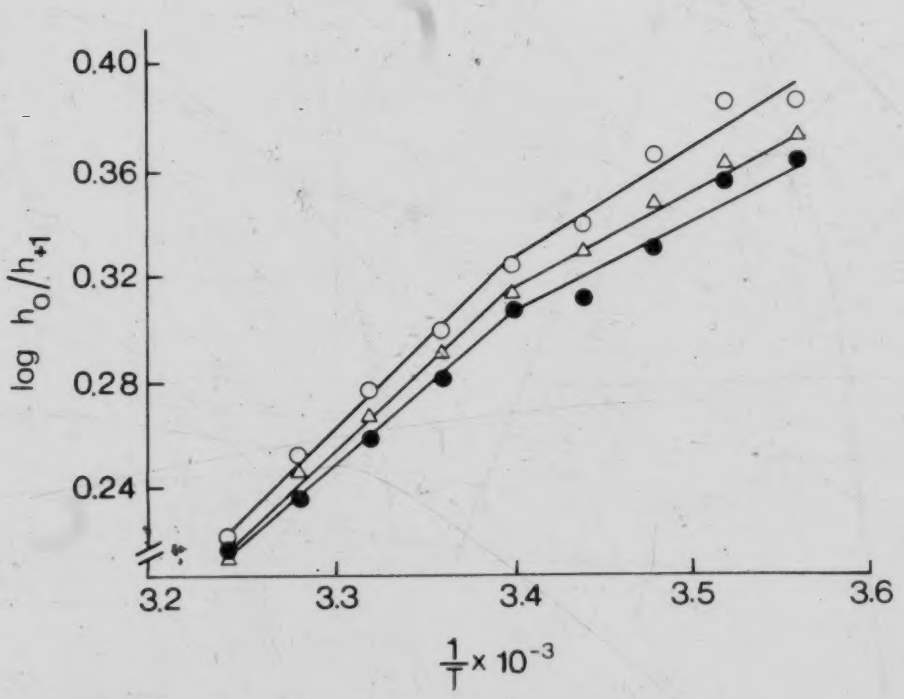

Fig. 26. A limited study of the effects of temperature and detergent extraction on restricted (R) sites labelled with M 12-NSE. The spin probe was allowed to diffuse into untreated ox brain membranes (△) and after extraction with sodium deoxycholate (○), or with sodium dodecyl sulfate (●), which were then equilibrated overnight at 4°C. About 30 μl of the labelled membranes were introduced into capillary tubes (1.1 mm internal diameter) which were then sealed. Temperature alteration was achieved by cooling the sample in multiple steps of a few degrees between 37° and 5°C. The plots represent mean values of 4 determinations for each condition. A single discontinuity near 20° can be seen under all experimental conditions.

Arrhenius plots of data from untreated microsomes again revealed the characteristic non-linearity that was observed with the data obtained in the more limited studies described immediately above. However as was discussed earlier (section 2.8, chapter II, page 41), the presentation of data solely as Arrhenius plots might also conceal secondary inflections. Consequently the data are presented in Fig. 27 as a simple linear plot of peak height ratios versus temperature.

The results suggest at least two characteristic temperatures at about 10°C and 20°C with a possible third transition near 30°C. In the latter case, the effect is seen more as a step between 28°C and 30°C, without any real change in the slope of the line between 22°C and 37°C. That is, there is no major alteration in the rate at which the mobility of the probe changes with temperature in this range. On the other hand, at 20°C there is a marked change in the rate of probe mobility which is seen as a change in the slope of the line between 10°C and 20°C, compared to that above this point. This reflects a temperature range in which the mobility of the probe is markedly reduced and provides the basis for the two-state concept described in the earlier studies which employed a less extensive number of data points. It is noted further, that at about 10°C there is an even more noticeable inflection point indicating a very marked loss of probe mobility below this temperature.

5.4 Extended study of the effects of temperature on R sites in DOC and SDS treated membranes.

Detergent treated preparations were also examined in similar detail. Again, simple linear plots of the peak height ratios versus temperature were drawn and are shown in Fig. 28. The data obtained

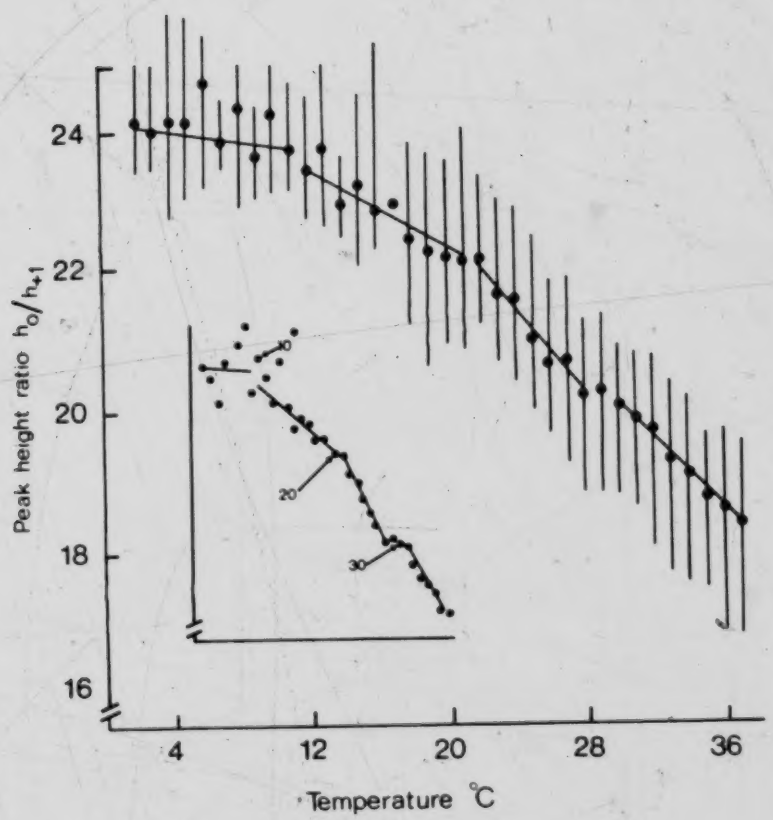

Fig. 27. A detailed study of the effects of temperature on spin labelled ox brain membranes. M 12-NSE was allowed to diffuse into membranes and was equilibrated overnight at 4°C. About 30 µl of labelled membrane were introduced into the sample tube which was sealed. Temperature alteration was achieved by progressively reducing the temperature of the sample chamber in intervals of 1°C, between 37°C and 2°C. The plot represents mean values of 4 determinations, with ranges. Multiple discontinuities can be seen near 10°, 20° and 30°C. These discontinuities are more marked in individual experiments and a typical plot is shown in the inset. The scatter below 10° (seen in the latter plot) may be due to "packing defects" (Lee, 1977).

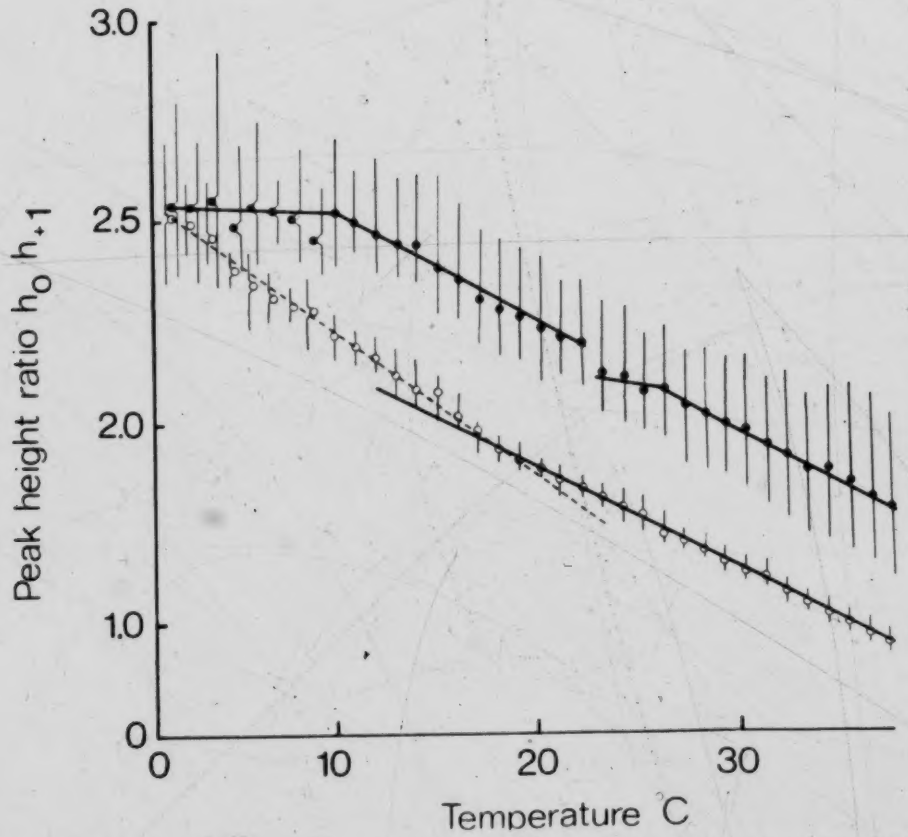

Fig. 28. A detailed study of the effects of temperature on spin labelled ox brain membranes. M 12-NSE was allowed to diffuse into membranes which had been treated with sodium deoxycholate (●) or with sodium dodecyl sulfate (○), and was equilibrated overnight at 4°C. About 30 µl of labelled membrane was introduced into the sample tube which was sealed. Temperature alteration was achieved by progressively reducing the temperature of the sample chamber in intervals of 1°C, between 37°C and 2°C. Both plots represent mean values of 4 determinations with ranges. Discontinuities near 10° and 26° are seen following extraction with sodium deoxycholate, whereas only a single discontinuity near 18°C occurs after extraction with sodium dodecyl sulfate.

after extraction with DOC are now different from those obtained with untreated enzyme preparations. From 37°C down to about 10°C there is no change in the slope of the line, although the presence of a step, now at about 25°C cannot be unequivocally excluded. The marked discontinuity at 10°C is still apparent. Thus on cooling DOC treated membranes, the changes in fluidity are more gradual and the marked discontinuity at about 20°C is eliminated.

This tendency towards a more homogeneous lipid matrix, is even more marked in the data obtained from SDS treated microsomes. Cooling of the labelled microsomes from 37°C down to 18°C now causes no interruption in the rate of change of probe mobility either in the form of a step or a change of slope in the plot. Below this temperature there is a small but detectable increase in the slope of the line which remains the same throughout the remainder of the temperature range that was examined. That is, there are only two states of probe mobility after treatment with SDS. This finding is essentially in agreement with the earlier results obtained from a limited number of data points. However, inspection of the present data suggests an inflection point at about 18°C rather than at 22°C as reported in the experiments described in Fig. 26.

5.5 Extended study of the effects of temperature on liposomes made from lipids extracted from ox brain $(Na^+ + K^+)$-ATPase.

From the experiments described above it is apparent that treatment of the membrane enzyme preparations with either DOC or SDS alters the response of the membrane lipids to changes in temperature. In order to further examine this aspect, the ESR method was applied to a

study of total lipid extracts of these preparations. Liposomes were made from these total lipid extracts and labelled with M 12-NSE. For comparison spin labelled liposomes were also made from ox brain phosphatidyl serine, a phospholipid that has been strongly implicated in the modulation of $(Na^+ + K^+)$-ATPase activity and temperature dependence (Schwartz *et al.*, 1975; Kimelberg, 1976).

The results are shown in Fig. 29 where it can be seen that there is a gradual increase in peak height ratio as the temperature is lowered. For both, the lipids of untreated microsomes and for liposomes made from ox brain phosphatidyl serine, this variation with temperature is readily described by two straight lines having significantly different slopes above and below intersection points at 20°C and 16°C respectively. With liposomes made from total lipid extracts of detergent treated microsomes, there is much less difference in the slopes of the lines above and below an inflection point at 20°C.

Of even more importance is the observation that the multiple discontinuities of untreated microsomes are reduced to a single inflection point in liposomes made from extracts of these preparations. Similarly the liposomes made from lipid extracts of detergent treated microsomes also differ from their parent preparations. The loss of these inflection points, strongly suggests a powerful co-operative effect by the enzyme protein on the physical behaviour of the lipid bilayer. More evidence for this co-operative effect is presented in the following section.

From the studies of the effects of temperature on spin labelled $(Na^+ + K^+)$-ATPase enriched membranes, the following summary of the

Fig. 29. A detailed study of the effects of temperature on spin labelled liposomes. M 12-NSE was allowed to diffuse into liposomes made from lipid extracts, during incubation for 10 min at 37°C. About 30µl of labelled liposomes were introduced into the sample tube which was sealed. Temperature alteration was achieved by progressively reducing the temperature of the sample chamber in intervals of 1°C, between 37°C and 2°C. The results obtained from lipid extracts of membranes treated with sodium deoxycholate (△) and sodium dodecyl sulfate (▲) were not significantly different and showed a single discontiniuity near 20°C. For comparison the results with a commercial preparation of ox brain phosphatidyl serine (o) and from lipid extracts of untreated ox brain membranes (●) are given below. Single discontinuities can be seen near 16°C and 20°C respectively. The results are the mean values of duplicate determinations.

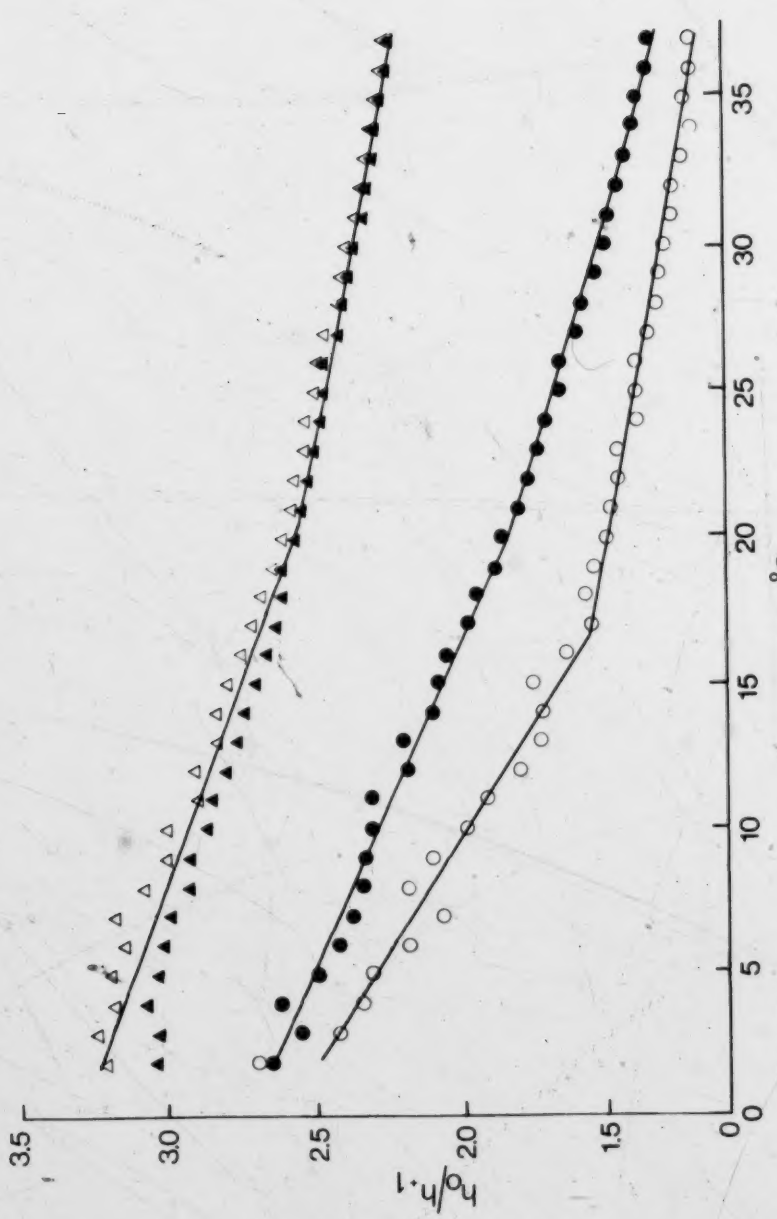

Fig. 29. Legend on separate page.

observations can be presented.

a) M 12-NSE at U sites report a sensitivity to temperature of the membrane which is apparently NOT accompanied by any thermal transitions of the membrane lipids. This result is sustained by membranes before and after extraction with DOC.

b) At R sites the spin label reports large changes in fluidity as the membrane is cooled. A limited study of 9 temperature points, when presented in the Arrhenius form, results in a typical plot with a discontinuity at about 22°C. Untreated membranes and those treated with DOC or with SDS are not markedly different either with respect to the inflection point or the slopes of the lines above and below Tc.

c) An extended study of the temperature sensitivity of R sites suggests three unique temperature points, at about 30°, 20° and 10°C, when analysed as a simple linear plot of peak height ratios versus temperature. Between 28°C and 30°C there is a step, but the rate of change of fluidity remains linear. At 20°C and 10°C the fluidity changes register discontinuities; as the temperature is reduced, the rate of loss of fluidity is progressively decreased.

d) Results from extended temperature studies of R sites on DOC-treated membranes differ from those of untreated membranes. The rate of change of fluidity is now linear over the range 37° to 10°C, with a marked decrease in this parameter below this temperature. The possibility of a step at about 25°C also exists.

e) Extended temperature studies of SDS-treated membranes differ from those of untreated membranes as well as those of DOC-treated membranes.

A single discontinuity at 18°C is observed. Above and below this temperature the change in fluidity is linear with the more rapid change manifested below 18°C.

f) Finally the results from extended temperature studies of liposomes made from lipid extracts of these three types of enzyme preparations are practically identical. There is a single discontinuity at 20°C, and the change in the fluidity of the membrane is more rapid below this temperature. There is a lack of compliance between sensitivity to temperature of the liposomes and the parent microsomes.

6. Extended study of the effects of temperature on the hydrolytic activity of ox brain ($Na^+ + K^+$)-ATPase.

The detailed ESR studies which have been described so far, have revealed hitherto undefined thermal characteristics in the behaviour of ($Na^+ + K^+$)-ATPase membranes; thus it was essential to re-examine the hydrolytic activity of the enzyme in the same detail. This was achieved by determining changes in ouabain-inhibitable enzyme activity at intervals of 1°C between 6°C and 37°C. Experiments with both detergent treated and untreated preparations were carried out. Fig. 30 shows Arrhenius plots of the data obtained from these experiments. In general the data from all three types of enzyme preparations are remarkably similar; in no instance can they be fitted adequately by a single straight line as there is a noticeable discontinuity at about 18°C.

The computer analysis for a two line fit was used to determine the apparent activation energies for the hydrolytic activity of the enzyme, above and below the critical temperature. The values of about 14 kcal/mole above, and about 30 kcal/mole below T_c are very similar

Fig. 30. A detailed study of the effects of temperature on enzyme activity before and after detergent extraction. The (Na^+ + K^+)-ATPase activity of untreated ox brain membranes (▲ ▲ ▲), and after extraction of the membranes with sodium deoxycholate (○ ○ ○), or with sodium dodecyl sulfate (● ● ●), was determined by a continuous assay procedure. For clarity the rates of enzyme activity which are given as μmoles Pi/mg protein/hr have been offset for the different preparations. At 37°C the specific activities of the preparations were 44, 125 and 95 μmoles Pi/mg protein/hr for the untreated, the sodium deoxycholate treated and the sodium dodecyl sulfate treated ox brain membranes respectively Multiple discontinuities are apparent in all preparations.

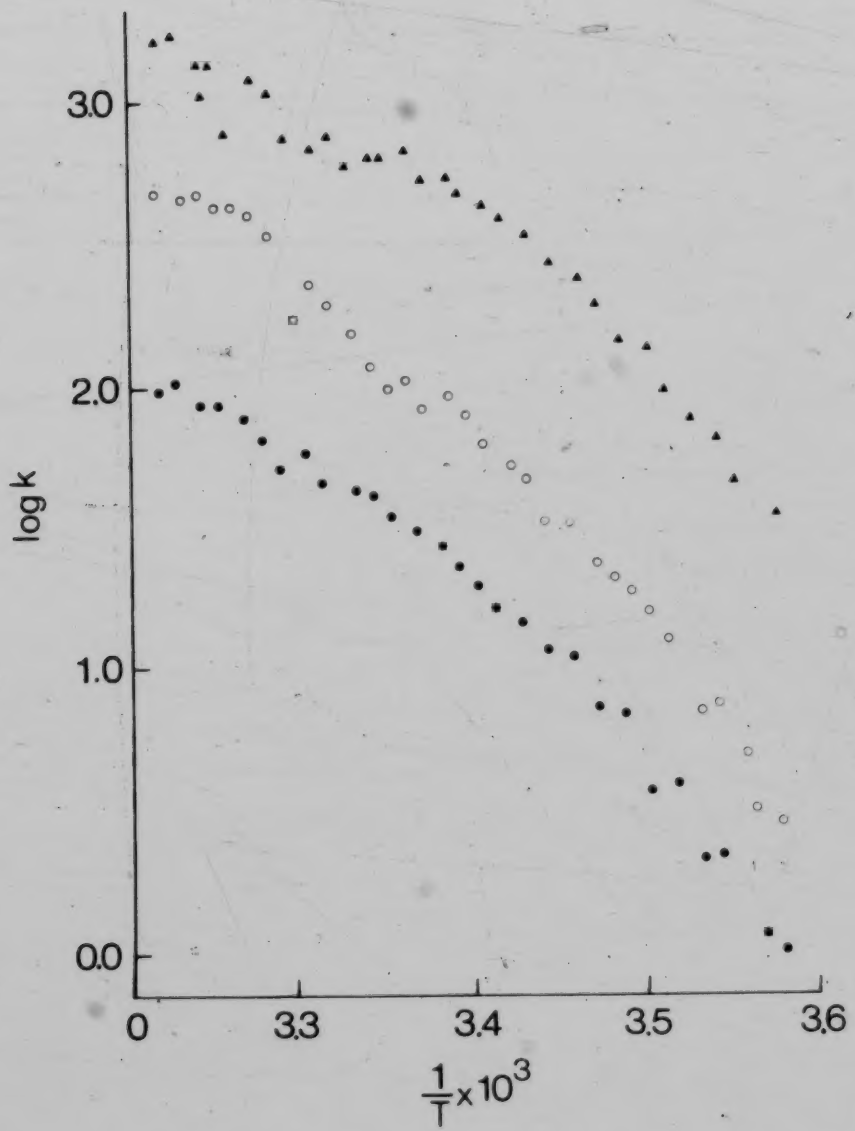

Fig. 30. Legend on separate page.

to the values obtained previously in the more limited studies carried out earlier, the results of which were given in Table 3, p. 75.

However, it seems very likely that other discontinuities which might correspond to the temperature characteristics observed in the ESR studies, also exist in this data, but are obscured by the method of analysis which only permits a single inflection point. When examined from this point of view all three plots can be shown to depart from linearity at several temperatures. There is a major step at 30°C, and a discontinuity at about 18°C. Below 6°C the rate of hydrolysis of ATP is markedly reduced and cannot be easily measured, due to technical limitations in the instrumentation; changes at low temperatures are therefore difficult to describe.

7. Spectral changes and membrane fluidity

7.1 Synthetic phospholipid bilayers.

The cooling of phospholipid bilayers leads to a decrease in fluidity. If a spin label is incorporated in the bilayer, then the reduced fluidity causes a loss of motional freedom of the nitroxyl reporter group. This effect was demonstrated in Chapter II, section 4.6, p. 56 using DMPC liposomes. Fig. 31 shows an illustration of an Arrhenius plot obtained from a typical experiment with spin labelled synthetic bilayers. As the bilayer is cooled there is at first a slow loss of fluidity, followed by a rapid change and again by a slow change. The rapid change in fluidity (the steep slope) reflects the transition of the lipids in the bilayer from a fluid phase to a gel phase, while the two discontinuities are the beginning and the end of this process (Lee, 1977). These changes can be interpreted to some degree at the molecular

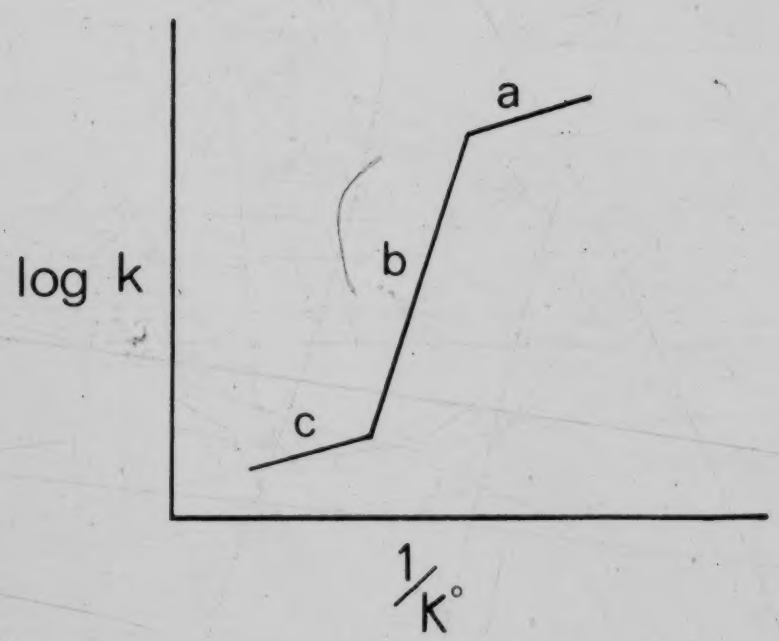

Fig. 31. Illustration of a typical Arrhenius plot of data obtained with spin labelled bilayers. An experimentally derived plot of this type can be found in Sackmann E., Trauble H., Hans-Joachim G., and Overath P., (1973) Biochemistry, 12, 5360.

level, but such a discussion would be inappropriate to this section, and will be found in a later section of this thesis.

Fig. 32 shows an Arrhenius plot of data obtained from spectral changes of DMPC liposomes labelled with M 12-NSE as they were cooled from 37°C to 18°C. As the bilayer is cooled there is at first a slow loss of fluidity which is followed by a sudden acceleration. Thus it is apparent that M 12-NSE reports the beginning of a phase transition in DMPC liposomes which occurs at about 21°C; the end of this phase could not be determined within the limited temperature range that was studied.

From the data in Fig. 32 the following observation is made. "The cooling of a bilayer leads to changes which occur at a slow rate before the onset of the phase transition which is followed by a fast rate of change during the phase transition". In the context of the spin label, these changes are manifested as a slow rate of decrease in motional freedom prior to the phase transition, followed by a faster rate during the phase transition. In the section that follows, this sequence of change in the motional freedom of a spin label, has been examined for ox brain microsomes labelled with M 12-NSE.

7.2 Spectral changes and membrane fluidity in ox brain microsomes.

The effects of temperature were first established for the enzyme activity of ox brain microsomes enriched with $(Na^+ + K^+)$-ATPase, before and after treatment with SDS. The results are shown in Fig. 33. The plot is typical of this enzyme with two distinct slopes representing different energies of activation above and below a critical temperature, Tc, such that the enzyme function is less efficient in the lower tempera-

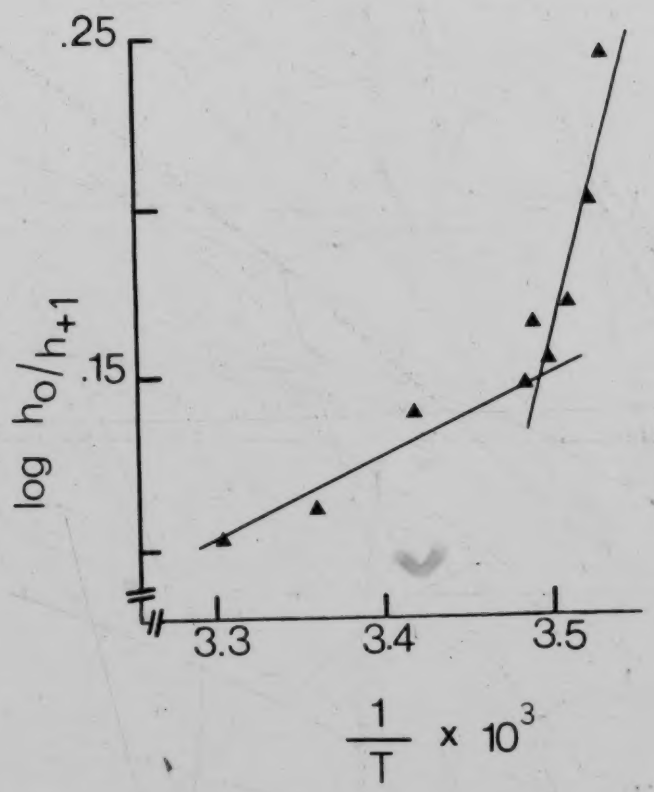

Fig. 32. Arrhenius plot of data obtained from M 12-NSE labelled DMPC liposomes. M 12-NSE was allowed to diffuse into DMPC liposomes which were then sampled for spectroscopic analysis. Temperature alteration was achieved by progressively reducing the temperature of the sample chamber between 37°C and 18°C. As the bilayer is cooled there is at first a slow loss of fluidity followed by a sudden acceleration of the changes in fluidity below about 20°C.

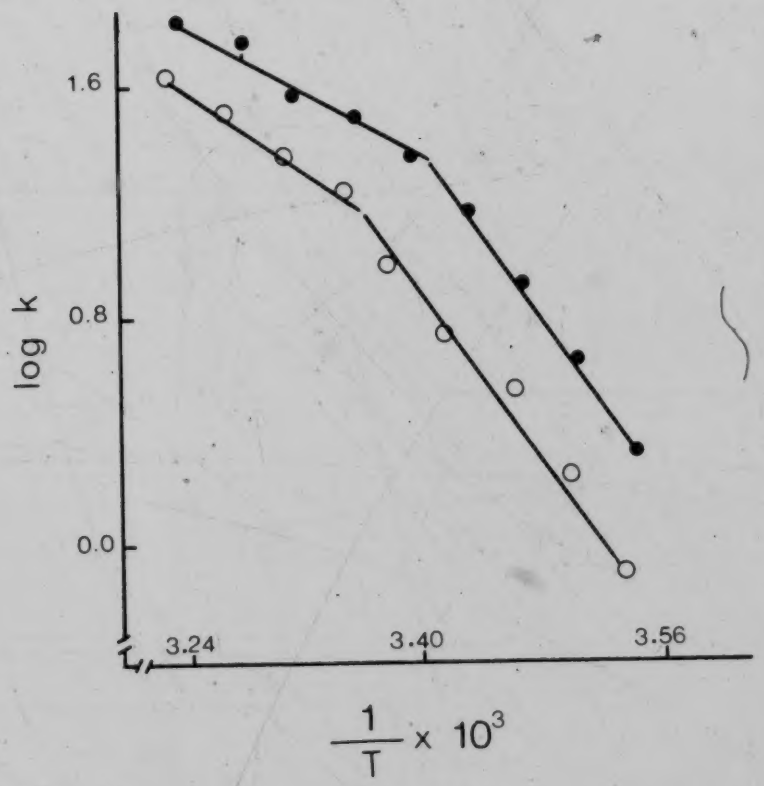

Fig. 33. Arrhenius plots of data obtained with $(Na^+ + K^+)$-ATPase enriched ox brain membranes, before and after extraction with sodium dodecyl sulfate. The $(Na^+ + K^+)$-ATPase activity of untreated ox brain membranes (○) and after extraction of the membranes with sodium dodecyl sulfate (●) was determined by a continuous assay procedure. Qualitatively sodium dodecyl sulfate extraction does not alter the biphasic thermal characteristics of this enzyme.

ture range; this information has often been demonstrated before (Charnock, 1978).

It is apparent that extraction with SDS does not alter the temperature sensitivity, despite the fact that detergent treatment markedly increases the specific activity of this enzyme (see appendix V).

Fig. 34 shows an Arrhenius plot of spectral changes reported by the lipid spin probe M-12-NSE intercalated in the ox brain microsomes. The microsomes were either cooled in jumps of a few degrees (saltatory cooling) or very gradually in 1°C intervals. The biphasic response is again clearly demonstrated and the value for Tc is also similar; the two slopes in these plots now reflect two distinct rates of change of molecular motion of the probe within the membrane, such that as the membrane is cooled below Tc there is a sudden decrease in the rate of change of its molecular motion. Both saltatory and gradual cooling produced identical results. ESR data were also obtained with SDS-treated enzyme preparations and are shown in Fig. 35. The two plots represent different rates of cooling. With saltatory cooling (open circles), the SDS-treated microsomes are identical to the untreated membranes. However, when SDS-treated membranes are gradually cooled (closed circles), a markedly different plot is obtained. Although the difference in slopes is now small, the biphasic nature of the plot is still apparent and the value for Tc is the same. A notable difference however is that the relative slopes of the two lines is now reversed: that is, the loss of probe mobility is more rapid below Tc. Thus it would appear that in SDS-treated membranes, the spin probe reports from two different environments.

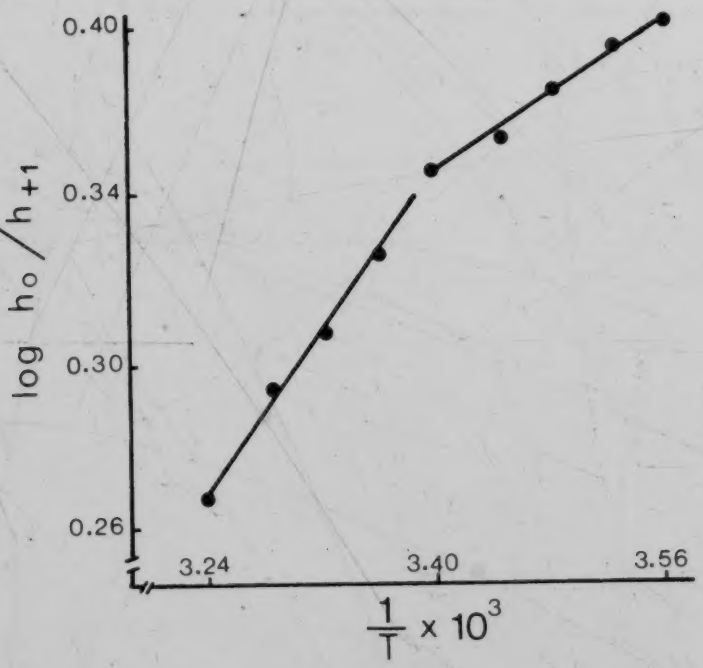

Fig. 34. Arrhenius plot of data obtained from ox brain membranes labelled with M 12-NSE. M 12-NSE was allowed to diffuse into ox brain membranes, and was equilibrated overnight at 4°C. About 30 µl of labelled membrane were introduced into the sample tube which was sealed. Temperature alteration was achieved by progressively reducing the temperature of the sample chamber, either in jumps of a few degrees (saltatory cooling), or gradually in 1°C intervals between 37°C and 8°C. The plot represents mean values of 4 determinations. The biphasic response of enzyme activity to temperature is duplicated by spectral changes of the probe M 12-NSE. Above Tc the changes in membrane fluidity are more rapid than below it. Saltatory or gradual cooling produced the same result.

Fig. 35. Arrhenius plot of data obtained from ox brain membranes labelled with M 12-NSE. M 12-NSE was allowed to diffuse into ox brain membranes that had been extracted with SDS, and was left to equilibrate overnightat 4°C. About 30 µl of labelled membrane were introduced into the sample tube which was sealed. Temperature alteration was achieved by progressively reducing the temperature of the sample chamber, either in jumps of a few degrees (○) that is saltatory cooling, or gradually in 1°C intervals (●). The plot represents mean values of 4 determinations. With saltatory cooling spectral changes of SDS treated membranes are identical to untreated membranes. With gradual cooling the sequence of the slopes is reversed; that is the change in membrane fluidity is fast below Tc and slow above it.

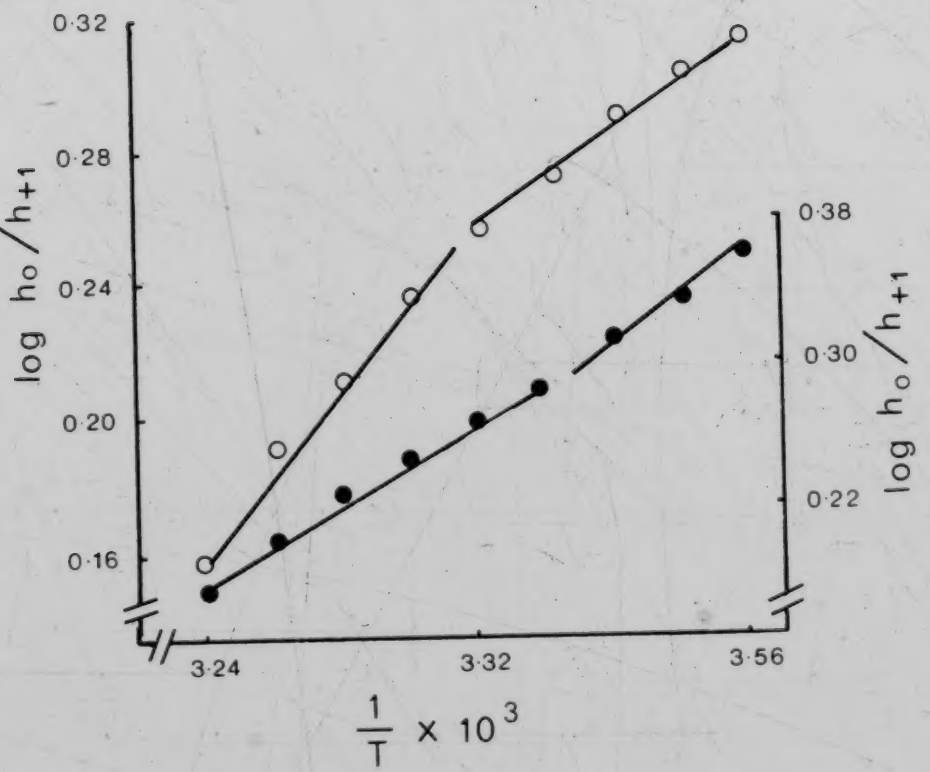

Fig. 35.

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From the results presented above, an interesting comparison can be made between microsomes and DMPC liposomes. On the one hand, untreated microsomes (irrespective of the cooling protocol), and SDS-treated microsomes subjected to saltatory cooling, both show a rapid loss of probe mobility above Tc and a slow loss below Tc; on the other hand with DMPC liposomes the sequence is reversed. To study this 'reversal phenomenon' further, liposomes made from $(Na^+ + K^+)$-ATPase-free total lipid extracts of these two types of membrane preparations were examined. The results are shown in Fig. 36. As was seen with the SDS-treated microsomes, the difference in slope above and below Tc is again small; nevertheless below Tc the probe reports a more rapid rate of change in its molecular motion. Moreover, there is no difference in the effects of temperature on the liposomes whether or not the membranes had been pretreated with SDS.

Thus from the data presented above, the following observations can be listed:

a) Purification of the enzyme by treating the membranes with SDS, does not alter its thermal sensitivity as determined by the hydrolytic activity.

b) The changes in molecular motion of the lipid spin probe M 12-NSE qualitatively duplicate the typical temperature phenomenon associated with the hydrolytic activity of this enzyme.

c) In spin labelled untreated microsomes, the rate of change in mobility of the probe is rapid at high temperatures, and gradual at low temperature. This result is the opposite of that found in synthetic phospholipid bilayers.

d) When SDS-treated microsomes are spin labelled, the rate of

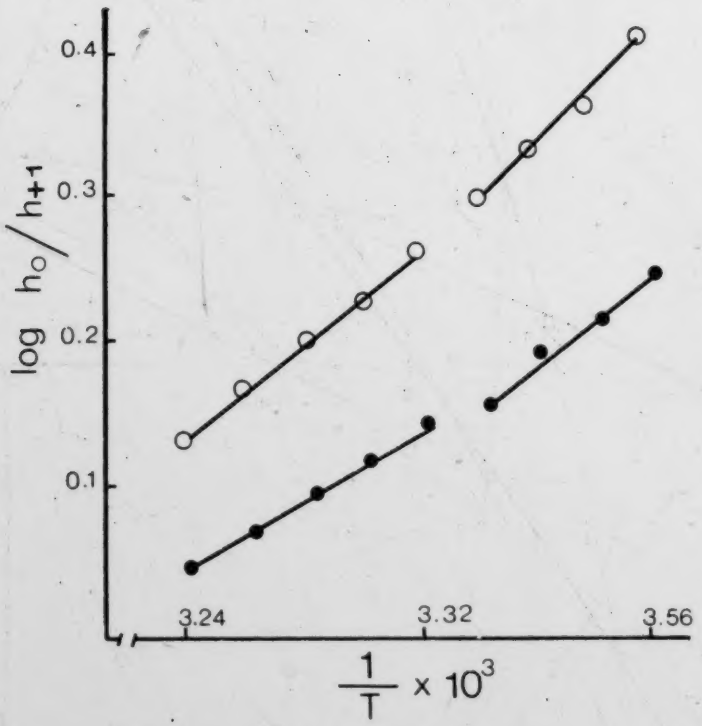


Fig. 36. Arrhenius plots of data obtained from spin labelled liposomes made from lipid extracts of ox brain membranes. M 12-NSE was allowed to diffuse into liposomes made from lipid extracts during incubation for 10 min at 37°C. About 30 µl of labelled liposomes were introduced into the sample tube which was sealed. Temperature alteration was achieved by saltatory or by gradual cooling of 1°C intervals between 37°C and 8°C. Liposomes made from extracts of untreated membranes (O) or from SDS treated membranes (●) show no difference. Above Tc the change in membrane fluidity is greater than below it. The cooling procedure that was used also did not alter the result.

change in mobility of the probe follows the profile of untreated microsomes with saltatory cooling; with slow gradual cooling, these treated microsomes behave like synthetic phospholipid bilayers.

e) In both the untreated and the detergent treated microsomes, the presence of the $(Na^+ + K^+)$-ATPase protein is essential for this atypical behaviour.

DISCUSSION

1. General

The project described in this thesis has examined two aspects of the $(Na^+ + K^+)$-ATPase, namely the binding of ouabain to the phosphorylated intermediate and the fluidity of the membrane in the physiological temperature range. The specific activity of the enzyme was used as an index for comparison of the two parameters mentioned above; purification of the enzyme by extraction with detergents or lipolysis with PPL-A were the treatments employed.

The specific activity of $(Na^+ + K^+)$-ATPase preparations can vary with the preparation. In addition, the preparation is usually 'contaminated' with a second ATPase which is insensitive to ouabain, the Mg^{++}-ATPase. In our laboratory, untreated enzyme preparations contain 30% or more of this second enzyme which by comparison with the distributions found in other preparations reported in the literature is relatively low. Therefore further purification is considered an essential step prior to study of the $(Na^+ + K^+)$-ATPase. To this end detergents are frequently employed although the precise mechanism by which they 'activate' the enzyme is not known. We have found that in SDS-treated preparations the contaminant enzyme is consistently reduced to less than 5% of the total ATPase activity. In addition to reducing the Mg^{++}-ATPase activity, detergents also serve to concentrate the $(Na^+ + K^+)$-ATPase, presumably by removing extraneous proteins and lipids.

In 1964 Ohnishi and Kawamura demonstrated the involvement of lipid in the hydrolysis of ATP by $(Na^+ + K^+)$-ATPase. Since then a vast body of information has accumulated which has provided valuable information about the $(Na^+ + K^+)$-ATPase itself and also the involvement of

the membrane lipids.

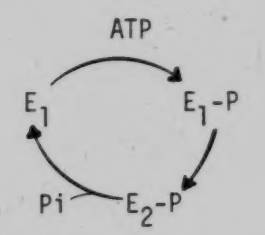
The use of the effects of temperature to assess the involvement of lipids in the biochemical function of this and other enzyme systems is frequently employed, and as a consequence the Arrhenius plot has become a useful tool.

2. The Arrhenius plot as a biochemical tool.

At its simplest level, the Arrhenius plot offers a means whereby the effects of temperature can be related to the reaction velocity in graphic form. From such a plot the activation energy, (Ea) can be calculated. In a number of biological reactions, the presentation of data in the Arrhenius form may result in a non-linear plot. In these situations an inflection point Tc can be derived and two energies of activation (Ea_1 and Ea_2) can be calculated above and below this point respectively. Such a situation pertains to the temperature activity relationships of the $(Na^+ + K^+)$-ATPase system; whereas a single inflection point has generally been accepted, the existence of more than one such temperature point has not been excluded. The importance of the Arrhenius plot to this dissertation necessitates some comment as to the implication of the Ea_1, the Ea_2 and the Tc in the function of the enzyme macromolecule.

The $(Na^+ + K^+)$-ATPase enzyme like a number of biological molecules functions optimally at 37°C *in vivo*. Therefore its activation energy must necessarily be assigned the value obtained from the slope of the line above Tc, that is Ea_1. This energy, which for native (untreated) ox brain membranes is about 17 kcal/mole, has not yet been assigned to any specific intermediate or partial reaction of the sys-

tem. From the reaction sequence usually depicted for this enzyme (p. 9), three steps could conceivably qualify as the energy limiting events and these are shown below:



The phosphate bond of ATP is known to be a high energy bond but the activation energy for E_1-P formation has yet to be determined. In the reaction sequence of the $(Na^+ + K^+)$-ATPase, this step is not thought to be the energy limiting step. The conversion of the E_1-P to the E_2-P intermediate necessitates a conformational change (Post *et al.*, 1969; Post *et al.*, 1972) by the protein macromolecule. According to Alber's group (Fahn *et al.*, 1966a; Fahn *et al.*, 1966b) the E_2-P form of the phosphointermediate represents a lower energy state than its predecessor E_1-P. This does not however exclude it from being the energy limiting step. The energy necessary for driving one conformation of a protein to another, could be energy limiting and still result in a product of lower energy.

The ultimate conversion of E_2-P back to E_1 and inorganic phosphate has not been examined extensively. The work in this area has been reviewed by Glynn and Karlish (1975). Although this reaction step is thought to occur spontaneously in the presence of water, there is also some suggestion that it is rate limiting (Mardh and Zetterqvist,

1972). Therefore, it must remain a contender for the energy limiting step.

Below Tc, the activation energy for this enzyme is increased two-fold. One theoretical explanation for this increase is that above and below Tc the sequence of conformational changes of the protein macromolecule may be different. That is, at the lower temperatures, the hydrolysis of ATP occurs by a different sequence and/or type of conformational change. Therefore, it is possible that the same partial reaction may still be the energy limiting step, and indeed may require different levels of energy depending on the temperature at which the reaction is carried out.

Another consideration for which there is much experimental support is the involvement of phospholipids in the operation of this membrane bound enzyme. Since the presence of some lipid/s is an absolute requirement for ATPase function, this possibility assumes importance. It is now well established that the lipid molecules of the membrane bilayer in which the enzyme is embedded are in a liquid-crystalline state above Tc and in the solid or gel phase below it. If lipids are involved in one or more of the partial reactions of (Na^+ + K^+)-ATPase then they could directly influence the activation energy of the enzyme. That is to say, the lipid contribution to the overall energy requirements of the (Na^+ + K^+)-ATPase might be reduced below Tc. If the information from the two slopes of an Arrhenius plot reflect the involvement of lipids in the energy requirements of the reaction mechanism, then three possibilities must be considered.

a) At temperatures below Tc, the lipids in their solid or gel state might hinder or retard the protein from attaining its

conformational forms.

b) Conversely, at temperatures above Tc the liquid crystalline state of the lipids might facilitate this process and thus reduce the energy requirements of the reaction sequence as a whole.

c) It is also possible that above as well as below Tc the protein and lipid components of the membrane bound enzyme obligatorily cooperate to achieve the process of ATP hydrolysis.

If the concept of hindering (case a) is correct then Arrhenius plots of partially delipidated enzyme preparations would show a decrease in the difference between the two activation energies. Ea_1 should remain unchanged while the value for Ea_2 should show a tendency towards approaching that of Ea_1. If facilitation (case b) is the explanation, then the situation would be reversed; the value for Ea_1 would be altered towards a value nearer Ea_2. Since the activation energy derived from linear plots is usually a value intermediate to Ea_1 and Ea_2 these two hypotheses do not seem to be likely. Therefore the third postulate, namely a co-operative protein-lipid effect must assume predominance in an interpretation of lipid involvement.

In the chapter on results section 7.1 a typical Arrhenius plot of a synthetic bilayer was illustrated (Fig. 31). From such a plot the temperature of the phase transition is readily obtained from the midpoint of the steep slope. If two lipids with differing transition temperatures are mixed then the transition temperature of the mixed bilayer is now a value intermediate to the individual values of the constituents. This is, the slope is less steep. In biological membranes, the phospholipids are usually a heterogeneous mixture with varying head groups, chain

length and chain saturation. Therefore, true phase transitions may not occur. Nevertheless biophysical techniques, such as those with spin probes (Grisham and Barnett, 1973) or fluorescent probes (Charnock and Bashford, 1975), have clearly established discontinuities in slopes obtained from temperature induced changes in the mobility of reporter molecules. These discontinuities may represent separations leading to the formation of clusters, or they may represent phase transitions of clusters or domains. For this reason in this dissertation Tc was defined as the temperature of the inflection or discontinuity and not the phase transition temperature.

3. Hydrolytic Activity of $(Na^+ + K^+)$-ATPase

The early studies of the effects of temperature on $(Na^+ + K^+)$-ATPase preparations from rabbit kidney cortex show that the enzyme displays the typical characteristics in that it yields a non-linear Arrhenius plot with distinct activation energies above and below a critical temperature. That is to say for the hydrolysis of ATP, this enzyme from rabbit tissue requires about 18 kcal/mole above and about 39 kcal/mole below a unique temperature, which from these studies is about 18°C. Furthermore, if the enzyme is treated with detergents such as DOC or Nonidet-P 40, these parameters do not change although the specific activity is markedly increased.

The two-fold increase in activation energy may hold information of some significance. In a recent review on 'membrane lipid phase-transitions', Charnock (1978) listed the activation energies for $(Na^+ + K^+)$-ATPase obtained from homeotherms, poikilotherms and hibernators. Although the values for Ea_1 from these preparations varied by 71%

(from 12.5 to 21.4 kcal/mole) and the corresponding variation for Ea_2 was 90% (from 23.7 to 45.1 kcal/mole), the ratio of these two energy requirements was consistently close to two-fold, with a mean ratio for Ea_2:Ea_1 of 2.1 and a range from 1.8 to 2.3.

Our later work with ($Na^+ + K^+$)-ATPase from ox brain shows that the thermal phenomena are very similar to those of the rabbit enzyme. The mean value for Ea_1 for untreated as well as detergent reated membranes was 17.0 kcal/mole, and for Ea_2 was 38.0 kcal/mole. Once again the activation energy is increased about two-fold below Tc, the ratio of Ea_2:Ea_1 being 2.2.

The results from studies of the hydrolytic activity of ox brain membranes were summarized in Table 3. Although the characteristic thermal phenomena are common to the different preparations and treatments (except PPL-A), some variation is apparent in the activation energies, both above (13.6 to 21.8 kcal/mole) as well as below (29.8 to 49.2 kcal/mole) Tc. By comparison the temperature of the inflection was less varied (16.6 to 20.9°C). It is not possible to explain these differences from what is currently known about this membrane bound enzyme. At the most, some tendencies can be pointed out.

For example, ($Na^+ + K^+$)-ATPase prepared from membranes that were homogenized with the polytron were usually more active than those prepared after disruption with the glass-teflon homogenizer. This increase in specific activity was accompanied by values for Ea_1 and Ea_2 that suggested greater fluidity. This tendency was also apparent with membranes that had been extracted with detergent. This mere hint makes a meagre contribution towards understanding the involvement of the lipids in ($Na^+ + K^+$)-ATPase function. Even SDS treatment which frequent-

ly yielded preparations with a 10-fold increase in specific activity, did not alter the biphasic nature of the Arrhenius plot or the inflection temperature. In fact the sharpest delineation of the discontinuous Arrhenius plot was often found following extraction with this detergent. Thus, although it is a reasonable assumption that tightly bound lipids must increase in parallel with the catalytic activity, our studies of the effects of temperature on the hydrolysis of ATP do not provide unequivocal evidence to support this assumption. In this regard it should be recalled that Wheeler *et al* (1975) were unable to show any marked change in phospholipid composition despite a 50-fold increase in specific activity of the membrane preparation after deoxycholate treatment and density-gradient centrifugation. Perhaps a better understanding is restricted by one or more of the limitations listed below:

a) The method used for determining the effects of temperature lacks the necessary precision.

b) The lipids are being concentrated in domains as a consequence of purification and their relative concentration is too small to have any marked effect on the thermal characteristics of the $(Na^+ + K^+)$-ATPase. The work from Wheeler's laboratory (1975) would certainly support this assumption.

c) The lipids being concentrated along with the protein macromolecule are not singularly responsible for the thermal phenomena associated with this enzyme, but jointly with the protein as well as the bulk lipid.

d) It is possible that the $(Na^+ + K^+)$-ATPase is capable of accommodating a degree of change in its membrane environment

without consequence to it's function. That is to say, changes may well occur in the composition of the membrane lipids on purification, but the protein-lipid interaction being dynamic in nature can adjust to accomodate the alteration.

The effects of temperature on the initial rate of [^{3}H]-ouabain binding to the enzyme revealed that like the ouabain-sensitive hydrolysis of ATP, an Arrhenius plot of the temperature-rate relationship was biphasic. However the difference in the apparent energies of activation above and below the inflection point was noticeably decreased. With preparations made by homogenization of the membranes with the polytron disintegrator, this tendency towards linearity was even more marked than when homogenization was carried out in a teflon-glass apparatus although the reason for this is not clear. If it is a consequence of the method of preparation then a loss of some lipid/s must result from the increased disruption, implying that the lipids associated with the binding site for ouabain are easily dislodged or altered in some way. This concept is supported by the fact that detergent treatments (DOC and SDS) which are without effect on the thermal sensitivity of the enzyme, readily abolish the effect of temperature on the initial rates of ouabain binding.

In addition, when untreated membranes prepared by disruption with a glass-teflon homogenizer were examined, non-linear Arrhenius plots were obtained whether or not the membranes were first extracted with DOC at 4°C. These observations do not agree with those of Siegel and Josephson (1972) or of Wallick and Schwartz (1974). However the

experiments by Siegel and Josephson (1972) were conducted after incubation for 15 minutes, which according to our very detailed study would only yield values for initial rates at the very low incubation temperatures in the range we examined or equilibrium levels only at the higher temperatures. In the report by Wallick and Schwartz (1974) the data points are too few to permit an analysis by the Bogartz technique used in this work (Cook and Charnock, 1978, in press); nevertheless on close inspection their published results might well be described as non-linear! Both these apparent discrepancies are discussed in greater detail in our recent publication on this subject (Charnock, Simonson and Almeida, 1977).

The rate of [^{3}H]-ouabain binding to PPL-A treated membranes yielded a linear Arrhenius plot. Interestingly, the addition of PS did not restore the non-linearity. This result is different from that obtained by ouabain inhibition of membrane ($Na^+ + K^+$)-ATPase activity that had been subjected to lipolysis with PPL-A. In the latter experiments, PS restored the non-linearity. Thus it appears that the binding of [^{3}H]-ouabain is less dependent on lipid than is the inhibition of the enzyme.

5. Lipolysis with phospholipase-A

In contrast to the effects of purification by detergent treatment, PPL-A has a dramatic effect on both the specific activity as well as the thermal phenomenon of the enzyme.

With previously untreated membranes, PPL-A first causes an increase in specific activity followed by a progressive decline to the untreated level. In the case of detergent treated membranes, which

have a higher starting specific activity, PPL-A did not cause any further increase in this index. Instead, the loss of activity on exposure to PPL-A with this agent was immediate, and progressed to the same level as that of the treated membranes. This distinction, namely the increase in catalytic activity by an agent which acts on the lipid and not on the protein (Hokin and Hexum, 1972), is not easily explained without invoking protein-lipid interactions. It is possible that the alteration of lipids in untreated membranes leads to a loss of non-enzymatic protein which in turn would result in larger numbers being obtained for specific activity without any true increase in the number of catalytic sites. In the case of detergent treated membranes, this loss of non-enzymatic protein would occur during the purification procedure. In other words, the distinction between untreated and treated membranes might only be a reflection of the inability of the assay procedure for protein content to distinguish between enzymatic and non-enzymatic protein molecules.

Alternatively, the increased activity could result from the exposure of latent sites by the lipase treatment. This latter suggestion was initially made for detergent activation of $(Na^+ + K^+)$-ATPase by Jorgensen and Skou (1971) and has since been supported by several other groups including our own laboratory (Charnock *et al.*, 1977).

When treated or untreated membranes were exposed to the action of PPL-A, a proportion of the catalytic sites were found to be resistant to the effects of membrane lipolysis. This observation is also not easily explained. Again it is possible that there are two distinct populations of catalytic units, the lipase resistant population being

considerably smaller in number. However, if it is recalled that the $(Na^+ + K^+)$-ATPase has an absolute requirement for lipid (Roelofsen *et al.*, 1971), then the lipase resistant population presumably has a singularly different lipid requirement. This interpretation would be supported by the frequently demonstrated result, the Arrhenius plots of this lipase treated enzyme are linear; the results of reactivation experiments with PS however, would not (Charnock *et al.*, 1975).

Temperature studies showed that delipidation of ox brain microsomes with PPL-A generated a marked change in the values of the two activation energies. Ea_1 shows an increase while Ea_2 decreases. This is unlike the tendency towards a more fluid membrane that was observed with detergent treatment. Thus whether these catalytic sites are new or not, the nature of lipid involvement in the catalytic activity is markedly altered as a consequence of PPL-A treatment. In addition, unlike our previous findings with $(Na^+ + K^+)$-ATPase from rabbit kidney cortex (Charnock *et al.*, 1973, 1975), and those of Taniguchi and Iida (1972) the specific activity when presented in the Arrhenius form did not now yield a linear plot. An inflection point could be detected at 30°C and although the two activation energies were not markedly different, their slopes were inverted. From the data it is not clear why this enzyme, after PPL-A treatment would be more efficient below the transition than above it, but it is of interest that this situation has been observed in a number of other biological situations where the source of the enzyme is an organism having unusual temperature requirements (Charnock, 1978).

Our observation (Charnock *et al.*, 1975) that the effects of

temperature on the cation activation of this enzyme are not altered by delipidation with PPL-A does not support the two site concept. Therefore, the possibility must be entertained that only a single population of catalytic units exists. Simpkins and Hokin (1973) have suggested that inactivation of $(Na^+ + K^+)$-ATPase by PPL-A may be due to a conformational change in the protein resulting from destabilization due to disruption of the phospholipid bilayer. If this is so then the residual enzyme activity may arise from catalytic sites functioning sub-optimally by employing alternate phospholipids in the membrane in a sub-optimal protein-lipid interaction. An interpretation such as this is favoured by the following information:

a) Residual catalytic units yield Arrhenius plots having a unique slope.

b) Some workers have achieved similar linear plots by extensive delipidation with detergents.

c) The effects of delipidation with PPL-A can be restored by PS.

6. Binding of ouabain to $(Na^+ + K^+)$-ATPase

The binding of ouabain to the $(Na^+ + K^+)$-ATPase was examined in three different types of membrane preparation, namely PPL-A treated, untreated and detergent treated, and the information will be discussed in this order.

As was observed with the experiments on the specific activity, the binding of ouabain to PPL-A treated membranes showed an increase initially followed by a followed by a progressive decline. If however the membranes were first purified with DOC then this biphasic response was no longer present. In contrast however, the binding of ouabain did

not decrease in parallel with the catalytic sites, whether or not the membranes were first purified with DOC. This result is in agreement with the earlier findings of Erdmann and Schoner (1973) but not in agreement with those of Goldman and Albers (1973), making further discussion necessary.

In discussing the increase in specific activity as a consequence of PPL-A or detergent treatment, the existence of two populations of catalytic units was introduced. Since the catalytic subunit of the enzyme macromolecule carries the phosphorylation site (as was documented in the introduction of this thesis) and ouabain binds to the E_2-P intermediate, it follows that the catalytic subunit also carries the ouabain binding site (Kyte, 1972a,b). Thus the concept of two populations of catalytic units must be extended to two populations of ouabain binding sites. As the catalytic sites increase after treatment with PPL-A or purification with DOC (exposure of latent catalytic subunits) so do the ouabain binding sites. However, further examination showed some discrepancies in this correlation, and this information is discussed below. As was presented in the chapter on Results, the experimental procedures enabled a study of the rate of ouabain binding as well as the amount of drug that was bound under equilibrium conditions. Thus, using the definitions of Erdmann and Schoner (1973, 1974) these experiments permitted a determination of both the affinity and also the number of ouabain binding sites. A summary of the data illustrating the effects of PPL-A on the specific activity and also on the affinity and number of ouabain binding sites was shown in Table 6 p.99. PPL-A decreased the specific activity to 15% of the pre-

treated control. In contrast the affinity of ouabain binding was reduced to 53% while the number of sites did not fall below 73%. So the catalytic activity exhibits a markedly greater sensitivity to the lipolytic effects of PPL-A than does the affinity or the number of ouabain binding sites. Furthermore, the affinity and the number of ouabain binding sites also exhibit distinct sensitivities to this lipolytic agent.

Therefore the credibility of the 'two population concept' that was described above is again called into question: if PPL-A causes destruction of a whole population of catalytic units, then the same effect is not observed either for the affinity or the number of drug receptors. That is to say, some of the catalytic units, despite having lost their catalytic activity still retain their capacity for binding ouabain. Nevertheless the observation is in agreement with the earlier work of Taniguchi and Iida (1973) and the recent conclusions of Hansen (1976) that two different types of ouabain binding sites are available in $(Na^+ + K^+)$-ATPase enriched membranes. Let it be emphasized however, that the alternate suggestion made above, of a single population of catalytic sites would also explain the findings. According to Simpkins and Hokin (1973) these sites are conformationally modified after PPL-A treatment.

If two populations of ouabain binding sites exist, the question arises as to whether these sites also exhibit multiple affinities. The data in Fig. 8A show that the rate of ouabain binding increases linearly with the specific activity, implying no change in the characteristics of the binding sites as the specific activity of the enzyme increases. However it was pointed out that the use of protein content as a reference

point for binding studies could obscure changes in affinity. By using a ratio of two rates which represent the catalytic and the drug binding properties of the enzyme, namely Pi release and ouabain binding, use of the protein concentration was avoided. The data shown in Fig. 9 p. employ this method of presentation. The absence of a line parallel to the abscissa permits the unequivocal statement, that "the affinity of the drug for the receptor is not constant over the range of specific activities that were examined" (10 to 158 μmoles Pi/mg protein/hr). It is also definite that the more pure enzyme preparations show an apparent decrease in affinity for the drug, which is in agreement with the findings of Lane *et al* (1973). These workers reported that a highly purified preparation of canine kidney ($Na^+ + K^+$)-ATPase bound [^{3}H]-ouabain at a slower rate than a relatively impure ($Na^+ + K^+$)-ATPase preparation from ox brain. However from the data presented in Fig. 9 it is not possible to categorically exclude the possibility of two distinct affinities, as opposed to a continuous gradual change.

The discussion of the 'two site' concept as it pertains to $(Na^+ + K^+)$-ATPase embraced a variety of information. In the following summary the salient features have been accumulated.

a) There are two populations of catalytic units, identifiable by activation with detergents or inactivation with PPL-A. When examined as an Arrhenius plot, the biochemical activity of these units as determined by ouabain inhibition does not differentiate between these two populations; however, after lipolysis with PPL-A, an agent which does not directly alter the structure of the protein macromolecule, some difference is apparent. In contrast, cation activation of the enzyme does not distinguish the two populations thereby recognizing only a single population. In addition, reactivation with PS after lipolysis with PPL-A also obscures or abolishes the difference.

b) There are two populations of ouabain binding sites, both of which reside on the catalytic subunit of the enzyme macromolecules. They apparently do not correspond either in number of PPL-A sensitivity to the catalytic units. The affinity of ouabain for the binding sites is not uniform. It is not known however whether the affinity has a binary distribution or whether a continuous variability exists.

Thus the 'two site' concept incorporates a number of observations, hypotheses and contradictions. In the opinion of this author a simpler model of a protein macromolecule with versatility of form and function might be a better concept. Such a model would avoid the rigidity of 'a site' and permit the advantage of a dynamic protein in a dynamic bilayer, at least until further information about this macromolecule becomes available. Therefore further investigation into the

properties of the single macromolecule would be more profitable than the demographic interpretation discussed above.

In the introduction, two questions were posed as being of importance to the study of ouabain binding to the E_2-P intermediate. One of these is repeated below:

> "Will the pharmacological actions of these drugs, namely the therapeutic and the toxic effects, be better understood by examining the binding of ouabain to the enzyme?"

Evidence has been presented that the affinity of the drug for the receptor decreases with purification of the enzyme. Furthermore, this effect might be consequent to the sequestration of specific lipids associated with the enzyme (as was shown from the ESR experiments). Taken together these observations provide a basis for future research into the toxicity of the cardiac glycosides. Can it be that toxicity, an important clinical aspect of these drugs bears some relationship to altered membrane lipids in the vicinity of the receptor?"

7. Electron spin resonance studies.

Although biophysical techniques such as fluorescence and ESR spectroscopy are extensively used to follow possible changes in the physical properties of lipids within biological membranes, it is not yet possible to precisely locate the reporter molecule. Thus spin labels may report from specific domains, from the bulk lipid, or from both (Jost *et al.*, 1973a,b). In addition, the spin probes may also dissolve in the aqueous solution and give rise to "liquid lines" (Giotta *et al.*, 1973; Butler *et al.*, 1974). The spin labelling method

described in this dissertation permits the direct labelling of biological membranes by diffusion of the spin probe M 12-NSE from the surface of the glass sample tube. Any residual label on the surface of the glass was shown to be of no consequence in the experiments that were conducted. It was also shown that the esterified form of this stearic acid spin label had a very low solubility in aqueous media making it possible to avoid any contribution to the spectrum from labels in solution.

This label readily diffused into liposomes made from DMPC, and these liposomes were used to show the effects of temperature on such bilayers, as well as to define the most appropriate method of spectral quantification. The results showed that M 12-NSE was an appropriate label for the study of fluidity changes in biological membranes. However when ox brain microsomes are spin labelled with M 12-NSE a variety of spectra may result. At high probe:membrane ratios the spectrum is isotropic, resembling that of a nitroxide reporter group tumbling freely in solution. At low probe:membrane ratios the spectrum becomes broadened and now resembles that of spin labelled liposomes. These two limiting cases are quite distinct and are best interpreted as originating from spin probes at two independent sites, an unrestricted site, designated as U and a site of considerably more restricted motion designated as R. Both U and R sites have been characterized to some degree. Labels at U sites are rapidly reduced by ascorbic acid and report a moderate change in fluidity on cooling the bilayer. Tumbling times were calculated (Raison *et al.*, 1971) for M 12-NSE at U sites, and were found to be similar to those for a water soluble spin probe in 0.25 M surcrose. Thus it is possible that U sites might be located at the membrane-water

interface. Such a position would comply with the rapid reduction of the spin label by ascorbic acid, but would require a bending of the polymethylene chain as described by Cadenhead (1975). Conversely, it is also possible that the nitroxyl resides in a very fluid area of the bilayer. Typical isotropic spectra such as those obtained with labelled U sites, have been described for spin labels in the lipid core of a model bilayer in the liquid crystalline state (Seelig, 1970). A hyperfine splitting value of 16 G was reported in this work by Seelig, and it compares favourably with 15.8 G, the value obtained from data described here.

Spin labels at R sites experience a marked increase in the viscosity of their environment as the temperature is reduced. The similarity of spectra obtained with labelled R sites and DMPC liposomes is indicative of a location deep in the hydrocarbon bilayer for these binding sites, and this is further supported by the lack of effect of ascorbic acid that was observed.

If both U and R sites on a membrane sample become labelled, then a mixed spectrum results. However, some experimental control of this mixture is possible. By adjusting the ratio of the probe to the amount of enzyme, the binding of M 12-NSE can be directed predominantly to one population of sites. Thus it is possible to examine either U sites or R sites with minimal interference from the other. This controlled labelling was of particular importance in the later work which was intended to examine the effects of temperature on the fluidity of $(Na^+ + K^+)$-ATPase enriched membranes.

The effect of membrane concentration on the spectrum varies from one enzyme preparation to another, and this variation can be correlated with the degree of purification of the membrane-bound

$(Na^+ + K^+)$-ATPase. This was especially noticeable with membranes that had been extracted with SDS. High activity SDS treated membranes appeared to be homogeneously labelled at the R sites. This correlation between R sites and specific activity of $(Na^+ + K^+)$-ATPase suggests a location for the R sites that is associated with the protein macromolecule.

The linear response to temperature of M 12-NSE at the so-called unrestricted or U sites lends further support to the suggestion made above, that this site is inherently very fluid whether it is at or near the membrane interface or deep within the membrane core. Wherever it is located, it is not influenced by thermal transitions within the membrane lipids, when examined by M 12-NSE, and is consequently of marginal interest to this work. However labelling at this site must be accounted for when interpreting data in subsequent studies.

On the other hand, ox brain microsomes labelled with M 12-NSE at restricted or R sites are markedly influenced by thermal transitions within the membrane. In our so-called limited studies which only employed nine observations within the temperature range 0° to 37°, the spin label reports fluidity changes with temperature that closely parallel the thermal sensitivity of $(Na^+ + K^+)$-ATPase. These results support the general conclusions of Grisham and Barnett (1973) who used spin labels and Charnock and Bashford (1975), who employed fluorescent membrane probes.

When the R sites are examined in studies with maximum data input that we could achieve and with minimal bias in the analysis, multiple temperature dependent inflections are revealed. For example, there is evidence that three such temperature points exist in microsomal enzyme preparations which have not been subjected to extraction by detergents.

It is singularly important to this work, that similarly detailed studies of the temperature-activity relationship of the hydrolytic activity of $(Na^+ + K^+)$-ATPase also reveals multiple discontinuities. Hence it can be seen that for untreated ox brain membranes the correlation between the thermal sensitivity of the enzyme and of the spin probe at R sites is fairly good.

As detergent extraction is applied to these preparations this level of correlation between ESR data and $(Na^+ + K^+)$-ATPase activity declines. For example some of the thermal effects upon the enzyme activity cannot be detected in either the DOC or in the SDS treated membranes. This is particularly noticeable in liposomes of the total lipid extracts of these preparations, all of which were devoid of multiple inflections, and could be characterized by a Tc at about 20°C. Liposomes from extracts of both detergent treated membranes, were also identical in their slopes above and below Tc, while the liposome preparations from extracts of untreated membranes showed the greatest overall change in fluidity with temperature, in the range 37°C to 3°C.

From the studies of the effects of temperature on spin labelled microsomes, it is apparent that the R sites report an increasing homogeneity in the membrane lipids as the enzyme is purified. These changes are in the order untreated < DOC-treated < SDS-treated preparations. Since the presence of residual detergent in the membrane would be an important factor in determining membrane fluidity, the preparations were checked after extraction with [^{14}C]-sodium deoxycholate and with sodium dodecyl [^{35}S]-sulphate followed by the usual washing procedures described under Methods. By using a value of 250,000 for the molecular weight of the $(Na^+ + K^+)$-ATPase macromolecule (Schwartz *et al.*, 1975) it

was calculated that there were less than 1.5 moles of residual DOC per mole ATPase in these preparations. It is unlikely that this could significantly alter the temperature response of the system. Conversely, the residual SDS was much higher, 150 moles/mole ATPase, and this amount of detergent could significantly influence membrane behaviour. However this interpretation is considerably weakened by the similarity with the results obtained from the liposome study of untreated microsomes which do not contain any detergent at all. While it is possible that the residual SDS may be acting as a significant fluidizer of the membrane lipids in general, this would again be in conflict with the results from the liposome study of the two detergent treated preparations.

The cholesterol content of the total lipid extract of all three types of membranes (untreated, DOC- and SDS-extracted) was also determined by a modified Liebermann-Burchard reaction (Huang *et al.*, 1961). In this study it was found that 16% of the total lipids (dry weight) was cholesterol in the untreated membranes, whereas after DOC and SDS, this value was reduced respectively to 14% and 11%. Whether this decreased cholesterol content coupled to the greater residual detergent that was detected accounts for the loss of correlation in results after SDS treatment cannot be determined from the data.

The results from ESR experiments discussed thus far emphasize three observations:

a) Increased specific activity after detergent treatment implies a concentration of this integral protein and presumably a concomitant increase of the associated lipids.

b) Enhancement of specific activity results in an increase in the number of R-binding sites in the bilayer for M 12-NSE.

c) As determined by the spin probe M 12-NSE the effects of temperature on the spectra of microsomes and liposomes is markedly dissimilar in untreated membranes, and less so after DOC treatment. After treatment with SDS however the correlation between microsomes and liposomes is good. This suggests an increasing homogeneity of the lipids in the order untreated, DOC treated and SDS treated.

d) It is unlikely that residual detergent in responsible for the altered behaviour of the lipids with changes in temperature.

So the question arises as to the nature of the phospholipid which is retained or concentrated in parallel with the protein macromolecule. It is recalled that brain tissue (the source of our enzyme) which is rich in $(Na^+ + K^+)$-ATPase, also contains proportionally higher concentrations of fatty acids with 18-C chains (Chapman, 1975). Therefore it must be considered that the suggested concentration or retention of lipids discussed above may occur by preference for characteristics of the methylene chain rather than by phospholipid head groups alone. This suggestion is also supported by work from other laboratories (Tanaka and Teruya, 1973) and in particular by the recent work of Palatini *et al.*, (1977). In their enzyme reconstitution experiments they showed that dimyristoyl phosphatidyl glycerol with its short chain fatty acids, and not dioleoyl phosphatidyl glycerol with the longer chains, resulted in a non-linear Arrhenius plot of $(Na^+ + K^+)$-ATPase activity.

The apparent lack of correlation between the effect of temperature on the liposomes made from total lipid extracts of the enzyme preparations and their parent micro-

somes clearly suggests an important ordering role for the protein on the lipid.

Thus, in addition to the selective retention of lipid species by the protein the suggestion is also made that the $(Na^+ + K^+)$-ATPase macromolecule, which is known to span the membrane, also exerts a powerful co-operative influence on the surrounding assembly of phospholipids.

Yet another possibility remains to be considered; after treatment with SDS the spin probe molecules might become bound to areas of lipid which are unrelated to the enzyme protein, and uninfluenced by the thermal behaviour of their phospholipid neighbours. Such an explanation however, would require migration of the spin labels from the binding sites in the untreated preparations since the latter clearly reflect similar thermal changes in both the hydrolytic activity of the enzyme protein and in the phospholipids of the membranes. Furthermore, a positive correlation between the incidence of R sites and enzyme purity was shown to exist for membrane preparations irrespective of the methods of purification. Thus it seem more likely that in the SDS treated preparations, the lipid "pool" containing the spin probe is adjacent to the enzyme, and whereas the fluidity of this region is reduced by cooling the methylene chains no longer become fully extended within this temperature range.

8. Membrane elasticity.

The preceding discussion has provided the basis for a model in which the co-operative effect between the $(Na^+ + K^+)$-ATPase protein and the lipids of the membrane is two fold. First, the protein macro-

molecule exerts an ordering effect on the phospholipids in the bilayer, and secondly, the alterations of the membrane fluidity caused by cooling of the fatty acid chains of the phospholipid in turn impose restraints on the protein macromolecule leading to conformational readjustment.

The data presented in the final section of the chapter on results (section 7) showed a relationship between the changes in membrane fluidity and in the spectrum of M 12-NSE in ox brain microsomes. From the data a number of observations were made and these are reproduced below:

a) Purification of the enzyme by treating the membranes with SDS, does not alter its thermal sensitivity of $(Na^+ + K^+)$-ATPase as determined by the hydrolytic activity.

b) The changes in molecular motion of the lipid spin probe M 12-NSE qualitatively duplicates the typical temperature phenomenon associated with the hydrolytic activity of this enzyme.

c) In spin labelled untreated microsomes, the rate of change in mobility of the probe is rapid at high temperatures, and gradual at low temperatures. This result is the opposite of that found in synthetic phospholipid bilayers.

d) When SDS-treated microsomes are spin labelled, the rate of change in mobility of the probe follows the profile of untreated microsomes, with saltatory cooling only; with slow gradual cooling which presumably allows time for some form of re-equilibration to occur between the probe and its environment these treated microsomes behave like synthetic phospholipid bilayers.

e) In both the untreated and the detergent treated preparations of the enzyme, the presence of the $(Na^+ + K^+)$-ATPase protein is essential for this atypical behaviour.

From these observations, the following conclusions can be drawn about $(Na^+ + K^+)$-ATPase containing membranes:

a) A physical change occurs in the microsomal membrane when it is cooled, which is not normally observed with bilayers made from synthetic phospholipids.

b) Since microsomes and not liposomes exhibit this 'unique' physical change, an interaction between the protein and its lipid environment must occur.

c) In SDS-treated membranes, the 'unique' physical change can be eliminated by gradual cooling, and may occur by a protein dependent sequestration of special phospholipids that have become concentrated along with the enzyme.

d) The conformational changes essential for enzyme activity respond to so-called phase transitions of the membrane lipids, irrespective of the degree of purification.

This information enables an extension of the concept of protein-lipid interaction as it pertains to the temperature sensitivity of $(Na^+ + K^+)$-ATPase.

Although well documented, the phase transitions reported for biological membranes are poorly understood. In 1967 Chapman *et al.* showed that the methylene chains of the fatty acids are involved in phase transitions. Above the transition the arrangement of the methylene chains is still uncertain, while below the transition the methylene

ILLUSTRATIONS OF FIGURES

Fig. 32

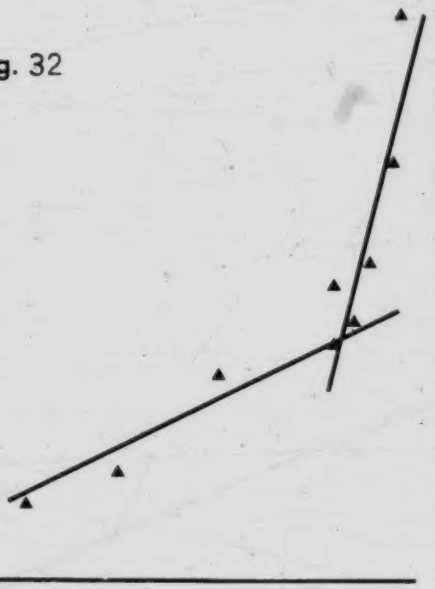


Fig. 33

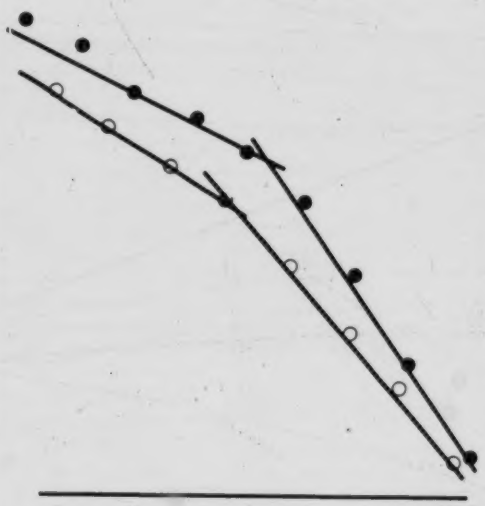
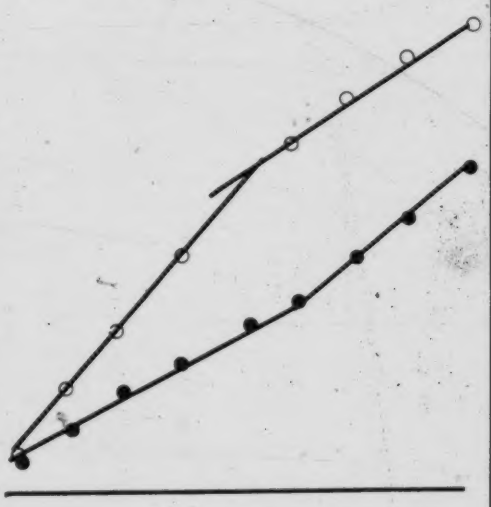


Fig. 34

Fig. 35



chains become stiff and lie parallel to each other and perpendicular to the plane of the membrane (Lee, 1977). The stiffening of the methylene chains due to cooling, causes an increase in width of the bilayer (Chapman *et al.*, 1967; Trauble and Haynes, 1971; Pagano *et al.*, 1973). Thus in Fig. 32, the more gradual slope between about 36°C and 21°C reflects a loss of mobility of the methylene chains above Tc, and the sudden steeper slope below 21°C probably reflects the stiffening of the methylene chains with a concommitant increase in width of the bilayer. It is reasonable to expect that a similar stiffening of the methylene chains accompanied by an increase in membrane width would also occur when biological membranes are cooled (Engleman, 1971). Therefore it seems most likely that when cooled, microsomal membranes reach a critical width at the Tc (Fig. 34) which leads to an interaction between the $(Na^+ + K^+)$-ATPase macromolecule and the phospholipids closely associated with it. This interaction opposes any further increase in this dimension. In the case of the SDS-treated microsomes (Fig. 35, open circles) a similar sequence of events occurs. If however, a slow cooling process is employed (Fig. 35, closed circles), the protein-lipid interaction permits an accumulation of lipid species which by virtue of their chain characteristics [length and(?) unsaturation] do not widen the membrane to the same degree.

In terms of the hydrolytic activity however, there is no difference in the effects of temperature on the untreated or detergent extracted preparations (Fig. 33). Thus, it is a reasonable presumption that the functional aspects of this pump-enzyme are acutely sensitive to changes in membrane width; when a critical membrane width is reached, the enzyme by adopting a new set of series of conformation changes main-

tains continued function, albeit with a decreased thermodynamic efficiency.

These results are compatible with a model in which the membrane fluidity which is essential for enzyme function, can exert its influence in two planes, parallel as well as perpendicular to that of the bilayer. This implies that the membrane behaves like a sheet of rubber with forces acting in the two planes. Weiss (1973) has proposed a "mechanicochemical principle" in his review on the role of lipid in energy transmission (Weiss, 1973). In his concept, Weiss hypothesized that "membrane receptors in lipid induce expansive tendencies at the surface of the membrane which are opposed by a spring-like expansive tendency from the contracted lipid". The concept presented here includes spring-like forces in more than one plane and permits the use of potential energy at any depth in the membrane. Inherent in this hypothesis is the concept that the membrane tends to return to its equilibrium state. It is conceivable then, that the conformational change from E_1-P to E_2-P by this transmembrane protein macromolecule, the $(Na^+ + K^+)$-ATPase, could cause an alteration in the balance of the two forces described above. Thus in the case of the $(Na^+ + K^+)$-ATPase enzyme system, it is likely that the protein moiety incorporates the potential energy of an elastic membrane in the energy transduction process that is necessary for transport.

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APPENDICES

APPENDIX I

PREVIOUSLY COPYRIGHTED MATERIAL,

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ACTIVATION ENERGY AND PHOSPHOLIPID REQUIREMENTS OF MEMBRANE-BOUND ADENOSINE TRIPHOSPHATASES.

J.S. CHARNOCK, D.A. COOK, A.F. ALMEIDA, AND REBECCA TO

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APPENDIX II

PREVIOUSLY COPYRIGHTED MATERIAL,

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TEMPERATURE-ACTIVITY RELATIONSHIPS OF CATION ACTIVATION AND OUABAIN INHIBITION OF $(Na^+ + K^+)$-ATPase

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APPENDIX III

APPENDIX III

Acetic acid, Glacial - Baker.

Acetic anhydride - Baker.

ATP-adenosine triphosphate, sodium. Grade 1 - Sigma.

Albumin, bovine serum - Sigma.

Calcium chloride - Fisher.

Chloroform - Fisher.

Cholesterol - Matheson, Coleman and Bell.

DOC-deoxycholic acid, sodium - Sigma.

DOC-^{14}C deoxycholic acid, sodium - Amersham/Searle.

Dimyristoyl phosphatidyl choline - Serdary Research Laboratories.

1,4-Dioxane, purified - Fisher.

EDTA - ethylene diamine tetic acetic acid - Fisher.

Glycylglycine - Sigma.

L-Histidine - Sigma.

Lactic dehydrogenase, Type II - Sigma.

Magnesium sulfate - Fisher.

Mercaptoethanol - Sigma.

Methanol - Fisher.

β-nicotinamide adenine dinucleotide, reduced form - Sigma.

Naphthalene - Fisher.

APPENDIX III (Cont'd)

Ouabain - Sigma.

PEP-phosphoenol pyruvate - Sigma.

Phosphatidyl serine - Serdary Research Laboratories.

PPL-A-phospholipase-A, bee venom - Sigma.

PPO(2,5-diphenyloxazole), Scintanalyzed - Fisher.

Potassium chloride - Baker.

Pyruvate kinase, Type II - Sigma.

Sodium chloride - Fisher.

SDS - sodium dodecyl sulfate - Sigma.

$SD^{35}S$-sodium - NEN.

Sodium sulfate - Fisher.

Sucrose - BDH Chemicals.

Sulfuric acid - Baker.

Tris base - Schwarz/Mann.

Toluene, Scintanalyzed - Fisher.

APPEMDIX IV

APPENDIX IV

Treatment of membranes with deoxycholic acid (DOC).

The earlier work on $(Na^+ + K^+)$-ATPase from this laboratory had been performed with a membrane preparation from rabbit renal cortex (see appendices I and II). As the ouabain binding experiments required larger amounts of enzyme than had hitherto been used, the rabbit kidney became an inadequate source of $(Na^+ + k^+)$-ATPase. For this reason it was replaced by the ox brain.

The first DOC treatment applied to the brain membranes was adapted from the earlier studies that employed rabbit kidney membranes. A three fold improvement in enzyme specific activity was achieved, from 15 to 49 μmoles Pi/mg protein/hr, and this is shown in Table 1.

Further purification was attempted by extracting the membranes with 0.1% DOC at 30° after protection with ATP. The protection of enzymes with ATP in the presence of detergents is extensively used at present and its effectiveness is not in any doubt. In spite of this a clear understanding of this phenomenon at the molecular level has not yet been achieved. It is possible that a particular protein conformation may be involved. For example, if the ATP-enzyme complex sustains a certain conformation of the protein, then it is conceivable that specific phospholipids may also be protected against removal by the detergent. For this reason an extraction with DOC in the presence of Na^+ and Mg^{++} was also attempted. The results are shown in Table 2.

It is noteworthy that the homogenisation procedure which was changed from a glass mortar with teflon pestle, to a polytron, has resulted in an increase in the specific activity of the untreated membranes.

Table 1.

	*Specific activity	%
Untreated	15	100
0.1% DOC, 4°, 10 min	49	327

Table 2.

Untreated	23	100
0.1% DOC,4mMATP,30°, 60 min	140	607
0.1% DOC,4mMATP,80mMNa,2mM Mg,30°,60 min	39	168
0.2% DOC, 4mMATP,80mMNa,2mMMg,30°,60 min	1	5

Table 3. Time study using 0.05% DOC,4mM ATP,80mM Na,2mM Mg, at 30°.

0 min	20	100
2	32	165
4	37	185
6	36	180
8	44	220
10	39	195

Table 4. Time study using 0.1% DOC,4mM ATP,80mM Na,2mM Mg, at 30°.

0 min	19	100
10	126	652
20	114	590
30	94	488
40	119	616
50	118	610

*Specific activity given in μmolesPi/mg protein/hour.

Extraction of the membranes with 0.1% DOC at 30° markedly improves the purification process, resulting in a six-fold increase in specific activity. This is not the case when the cations Na^+ and Mg^{++} are present. With 0.1% DOC less than a two-fold improvement results and if the DOC is increased to 0.2% then the enzyme activity is lost. Thus it appears that when cations are present the enzyme is more labile, and the optimal concentrations of DOC and/or exposure time, is altered.

Table 3 shows a time study of the effects of 0.05% DOC on membranes pretreated with 4mM ATP in the presence of 80mM Na^+ and 2mM Mg^{++}. From the data in this table it is apparent that this low concentration of DOC again produces a relatively small increase in the specific activity of the enzyme. Only a two-fold increase is obtained after an exposure time of 8 min. On comparison with the data shown in Table 2, it seems possible that a better purification might be achieved with 0.1% DOC and a shorter exposure time. This possibility was examined, and the results are shown in Table 4.

The data show that about a six-fold purification is the maximum that can be achieved under these conditions, and it is attained within 10 min of addition of the detergent. Quite clearly the presence or absence of cations, the concentration of DOC and the exposure time are not the only factors controlling the degree of purification of these membranes. Subsequent purification employing 0.1% DOC and 4mM ATP, showed little change after 30 to 35 min and up to 60 min. Since the attainment of ideal conditions for purification was not essential to routine procedure hereafter was a 40-minute exposure to 0.1% DOC at 30° after the enzyme had been protected with 4mM ATP.

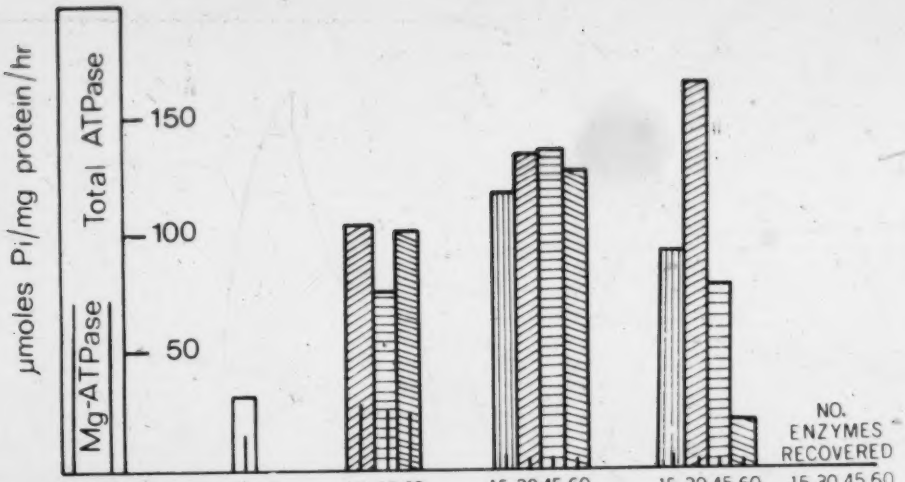
APPENDIX V

APPENDIX V

The histogram shows the effect of two variables on $(Na^+ + K^+)$-ATPase activity, namely SDS/protein ratio and the exposure time. The following observations can be drawn.

1. There is a gradual increase in the specific activity of the enzyme in response to the concentration of SDS.
2. At the higher SDS concentrations, solubilization of the protein follows purification of the $(Na^+ + K^+)$-ATPase. This results in a decreased purity as well as a loss of recovery.
3. Optimal purification may result on treatment of the enzyme with 0.1% SDS, but at this concentration the exposure time becomes very critical. Therefore as a routine procedure it is unsuitable.
4. Treatment of the enzyme with 0.05% SDS results in about a four-fold purification over a broad exposure time of about 30 to 45 minutes.

From this study the routine SDS purification procedure employed a 30-minute exposure of 1mg protein/ml to 0.05% solution of SDS. Usually a five-fold purification was observed but the value varied from not less than 4-fold to a use of 8-fold.



OUABAIN SENSITIVITY	68	73 68 75	97	98	
SDS mg / PROTEIN mg	–	0.2	0.5	1.0	3.0

APPENDIX VI

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VARIATION IN SENSITIVITY OF THE CARDIAC GLYCOSIDE RECEPTOR CHARACTERISTICS OF $(Na^+ + K^+)$-ATPase TO LIPOLYSIS AND TEMPERATURE.

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APPENDIX VII

PREVIOUSLY COPYRIGHTED MATERIAL,

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AN ELECTRON SPIN PROBE STUDY OF $(Na^+ + K^+)$-ATPase-CONTAINING MEMBRANES.

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